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PHYSIOLOGICAL STUDIES IN RELATION TO DEVELOPMENT AND GERMINATION OF SCLEROTIA OF *SCLEROTINIA SCLEROTIORUM*

by

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A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "PHYSIOLOGICAL STUDIES IN RELATION TO DEVELOPMENT AND GERMINATION OF SCLEROTIA OF SCLEROTINIA SCLEROTIORUM" submitted by James Rodney Letal in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

Physiological studies of mycelium, and development and germination of sclerotia of Sclerotinia sclerotiorum were carried out. As well, germination of sclerotia of two isolates of S. borealis was studied.

Yields of mycelium and sclerotia of S. sclerotiorum cultured on liquid malt-yeast medium were determined. Except for young sclerotia, the fresh and dry weights of sclerotia per petri plate were higher than the fresh and dry weights of mycelium respectively.

Total soluble proteins for mycelium, ungerminated sclerotia, and germinated sclerotia with the apothecia removed were determined. The protein content, on a colony basis, was higher in sclerotia than in mycelium; however, on a dry weight basis protein content was higher in mycelium.

Using disc gel electrophoresis, the protein patterns were obtained for ungerminated sclerotia of various ages, germinated sclerotia with the apothecia removed, and apothecia of S. sclerotiorum. The protein patterns of sclerotia varied with age of culture and medium used. The protein patterns of mature ungerminated sclerotia, germinated sclerotia with the apothecia removed, and apothecia of two isolates of S. borealis were also determined using disc gel electrophoresis. There were differences in protein patterns of mature sclerotia between S. sclerotiorum and two S. borealis isolates; however, no differences in protein patterns of sclerotia were observed between the two S. borealis isolates. The protein patterns of apothecia for all three isolates were different.

Peroxidase and polyphenol oxidase isozymes of ungerminated sclerotia, germinated sclerotia with the apothecia removed, and apothecia of S. sclerotiorum were also separated by disc gel electrophoresis. The patterns of these isozymes in sclerotia were also shown to vary with age of culture. The isozyme patterns for apothecia differed from those of sclerotia.

The effect of plant extracts, glucose, 1,3-dichloropropene, and light and dark treatments on apothecia production were determined. The plant extracts had a greater effect on apothecia production if they were present during the formation of sclerotia than if they were present during germination of sclerotia; glucose concentration during the growth of sclerotia was important in subsequent apothecia production; 1,3-dichloropropene treatments of mature sclerotia increased apothecia production; and sclerotia germinated in the light produced mature apothecia, whereas those germinated in the dark only produced apothecial stipes. Regardless of treatment of sclerotia mature apothecia were not produced in the dark.

Of a number of compounds tested, sodium diethyldithiocarbamate was the only one which inhibited pigmentation of developing sclerotia.

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INTRODUCTION AND LITERATURE REVIEW

Sclerotinia sclerotiorum (Lib.) DeBary was first called Peziza sclerotiorum by Madame Libert in 1837. In 1869, this fungus was transferred to the genus Sclerotinia (Wakefield 1924). In his synopsis of genera and species of Sclerotiniaceae, Whetzel (1945) referred to this fungus as Sclerotinia sclerotiorum (Lib.) DeBary.

Considerable work has been done on the growth and morphological aspects of S. sclerotiorum (DeBary 1887, Ramsey 1925, Henson 1935, Keay 1940, Henson and Valleau 1940, Purdy 1955, Arimura and Kihara 1968). In addition, numerous studies have been made on the nutritional factors affecting growth of this fungus (Newton 1946, Tanrikut and Vaughan 1951, Démétriadès 1952, Démétriadès 1953, Purdy and Grogan 1954, Bedi 1956, Bedi 1962, Willis 1968). Recent studies by LeTourneau (1966) and Cooke (1969) were on the carbohydrate content of sclerotia of S. sclerotiorum. Cooke (1969) also analysed the exudate which appears on young sclerotia for carbohydrates. Factors affecting apothecia were also studied (Young 1934, Young 1936, Keay 1939, Henson and Valleau 1940, Bedi 1956, Purdy 1956, Bedi 1962, Bedi 1963, Williams and Western 1965).

Little or no other work has been done on the biochemical changes occurring during the different stages of growth and development of this fungus. For this reason, biochemical studies during the different stages of this fungus were carried out. This included growth studies, total protein analyses of mycelium and sclerotia, protein studies using disc gel electrophoresis, amino acid analyses of sclerotia

and apothecia, factors affecting apothecia production, and studies on the pigmentation of sclerotia.

The separation of proteins extracted from fungal mycelium using starch gels (Clare 1963, Clare and Zentmyer 1966, Hall 1967) and polyacrylamide gels (Durbin 1966, Gottlieb and Hepden 1966, Hall *et al.* 1969, Glynn and Reid 1969) has been used principally for taxonomic purposes. Whitney *et al.* (1968), Sekhon and Colotelo (1968) and Zafar (1970) found that the protein patterns varied with age of culture. However no work has been done comparing the protein patterns of extracts from different stages of growth of fungi leading to the production of a sexual fruiting structure. The major portion of the following research is on the protein patterns of mycelium at various ages, sclerotia at various ages, and apothecia of *S. sclerotiorum* as obtained on polyacrylamide gels by disc gel electrophoresis.

The only studies available on the growth of *Sclerotinia borealis* Bubak and Vleugel are those by Cormack and Lebeau (1939), Groves and Bowerman (1955), Sprague *et al.* (1961), and Ward (1966). Groves and Bowerman (1955) refer to this fungus as *S. borealis* Bubak and Vleugal but Sprague *et al.* (1961) and Ward (1966) refer to it as *S. borealis* Bub. and Vleug. In the following research, *S. borealis* is compared to *S. sclerotiorum* as to apothecia production (including sizes of ascospores and asci) and protein patterns of buffer soluble proteins of sclerotia and apothecia.

MATERIALS AND METHODS

A. FUNGAL ISOLATES

The fungi used in these studies were Sclerotinia sclerotiorum (Lib.) deBary, and two isolates which were tentatively identified as Sclerotinia borealis Bubak and Vleugel. The S. sclerotiorum culture was grown from sclerotia removed from infected sunflower collected at Redwater, Alberta. The S. borealis cultures were grown from sclerotia removed from infected unidentified pasture grass and infected fall rye collected at Smokey Lake, Alberta and Beaverlodge, Alberta, respectively. These two isolates were designated as Sb_3 (pasture grass) and Sb_4 (fall rye).

B. GROWTH STUDIES

The stock cultures of these fungi were maintained on potato dextrose agar (PDA) at 3-5°C. Working cultures were maintained on a malt-yeast agar medium (Ward 1966). This medium was composed of Difco malt extract, 15 g; Difco yeast extract, 15 g; dextrose, 45 g; agar, 17.5 g; and distilled H_2O to 1 l. This medium was sterilized by autoclaving for 15 minutes at 121°C and 17# pressure. Thirty ml of this medium was added per each 90 x 20 mm petri plate. The inoculum consisted of 7 mm plugs of mycelium taken from the periphery of growing colonies, or sclerotia which were placed in the center of the above petri plates.

S. sclerotiorum cultures were grown at 20°C since this temperature was found to be the optimum for the growth of this fungus (Tanrikut and Vaughan 1951). The Sb_3 and the Sb_4 cultures were incubated at 10°C. This temperature was found to be the optimum for the growth of these

isolates (Colotelo, unpublished data).

(a) Wet and dry weight determinations of mycelium and sclerotia

The fungus S. sclerotiorum was grown on a liquid malt-yeast medium[1]. A 40 ml aliquot of this medium was added to each 90 x 20 mm petri plate and the medium was sterilized by autoclaving. Inoculum plugs (7 mm diam.) taken from the periphery of 4-day old colonies were placed in the petri plates containing the above cooled medium. Care was taken that the inoculum plug was in the center of the petri plates during the incubation period. The cultures were incubated at 20°C.

Wet and dry weights of mycelium from 4-, 5-, 6-, 7-, 8-, 9-, 10-, 15-, 20-, 25-, and 30-day old cultures were determined. Except for the 4-day old cultures, since sclerotia had not yet formed at this time, the wet and dry weights of sclerotia were determined for the above-aged cultures.

The sclerotia were separated from the mycelium using a sharp scalpel. The excess moisture on the surface of the mycelial mat and sclerotia was removed with paper towels prior to obtaining the wet weights. The mycelial mat and sclerotia from each petri plate respectively were placed on tared filter papers and dried at 90°C for 24 hours for the dry weight determinations.

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[1]This medium, with the agar excluded, was the same as that described on page 3.

(b) Apothecia production

(i) Apothecial initials were produced from sclerotia of S. sclerotiorum using the method of Purdy (1956). In this procedure sclerotia from 60- to 90-day cultures were partially submerged in sterile H_2O and incubated at 15-18°C.

Preliminary experiments using the liquid malt-yeast medium and a synthetic medium[2] indicated that apothecia would form from sclerotia grown on the synthetic medium but not from sclerotia grown on the liquid malt-yeast medium. The temperature of incubation for the cultures grown on the liquid malt-yeast medium was 20°C and 15°C for the cultures grown on the synthetic-agar medium. Note: during the later part of these studies it was found that apothecia could be produced from sclerotia removed from cultures which were incubated at 15°C and grown on the malt-yeast-agar medium. Sclerotia from 70-day old cultures, grown on the synthetic medium at 15°C, were placed into petri plates containing 20 ml of sterile, distilled and deionized H_2O and incubated at 15°C in the light. The light source was two 16" fluorescent bulbs placed approximately 20 cm above the petri plates. At this distance 180-220 ft. candles of light were measured using a Weston illumination meter, model 756. This form of light was used since Lane and Sproston (1955), Purdy (1956), and Bedi (1962) found that either artificial or natural light could be used for producing expanded apothecial initials.

The next stage in development was to determine whether asci and ascospores were found in the expanded apothecia. Portions of the

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[2]This synthetic medium, excluding the vitamins, was prepared as described by Ward and Colotelo (1960).

crushed apothecia were stained with cotton blue-lactophenol[3]. Photographs were taken using a Leitz Wetzlar ortholux microscope and measurements of apothecia, asci, and ascospores were taken.

(ii) For isolates Sb_3 and Sb_4 a slightly different procedure was used to produce apothecia. Sclerotia removed from 165-day old cultures incubated at 10°C and grown on the malt-yeast-agar medium were partially submerged in distilled and deionized H_2O and were incubated at 10°C in the light. As with S. sclerotiorum, a light intensity of 180-220 ft. candles was maintained at the petri plate level during incubation.

Portions of crushed apothecia stained with cotton blue-lactophenol showing paraphyses and asci containing ascospores were photographed as above, and measurements were taken.

In conjunction with the above growth studies, which included production of mycelium, sclerotia, and apothecia, biochemical analyses, principally protein changes which occurred during growth and differentiation, were carried out.

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[3]This stain was composed of aniline blue ws (cotton blue), 1 g; glycerol, 25 ml; lactic acid, 25 ml; phenol, 25 g; and distilled and deionized H_2O, 25 ml (Peacock 1966).

C. SOLUBLE PROTEINS

The total soluble proteins of mycelium and sclerotia of S. sclerotiorum were extracted from 4-, 5-, 6-, 7-, 8-, 9-, 10-, 15-, 20-, 25-, and 30-day old cultures grown on the liquid malt-yeast medium. The procedure used during extraction was as follows. Mycelium and sclerotia were separated and the liquid associated with the mycelial mat and sclerotia was removed using paper towels. The fresh weight of the mycelial mat from one petri plate was taken. This mat was placed into a plastic centrifuge tube (1.5 cm in diam.) to which was added 2.0 ml of phosphate buffer[4] per gram fresh weight of mycelium. This material was frozen at -48°C for 24 hours. The fresh weight of sclerotia from one petri plate was also taken after which the sclerotia were ground in a Krups grinder, model 308-60c. One gram fresh weight of the ground sclerotial material was placed in a similar plastic centrifuge tube and again 2.0 ml of the phosphate buffer was added per gram fresh weight of material. This sample was also frozen at -48°C for 24 hours. After disruption of the cells, using a chilled Hughe's press, the mycelial and sclerotial samples were treated in a similar manner. The disrupted tissue was placed into a centrifuge tube to which was added another 2 ml aliquot of the above phosphate buffer per gram fresh weight of tissue. The resulting slurry was centrifuged at 24,000 x g for 15 min. The supernatant was decanted and the pellet was mixed with an aliquot (as above) of the phosphate buffer. This slurry was also centrifuged

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[4] 0.1 M phosphate buffer, pH 6.0 was used. This buffer was prepared by adding 2.3772 g KH_2PO_4 and 0.4285 g K_2HPO_4 to 200 ml distilled and deionized H_2O. The buffer was stored at 3-5°C.

at 24,000 x g for 15 min. After decanting the supernatant, this extraction procedure was again repeated.

The supernatants from centrifugation were combined and thoroughly mixed by shaking. Two 50-ul aliquots of this supernatant were used for total protein determinations. Quantitative amounts of protein were obtained by comparing the results of the fungal samples to that obtained using standard amounts of bovine serum albumen (Nutr. Biochem. Corp.). Two readings were obtained for each sample and the experiment was repeated once. The procedure used for total protein determinations was essentially that of Lowry et al. (1951) but with minor modifications. Details of the procedure are as follows. Duplicate samples containing 0, 25, 50, 100, 200, 300, 400, and 500 ug per ml of the bovine serum albumen in the above phosphate buffer were used to prepare the standard curve. The volumes were made up to 5.0 ml with H_2O. To each sample was added 5 ml of a solution containing 50 ml of 2% Na_2CO_4 in 0.1 N NaOH and 1 ml of 0.5% $CuSO_4 \cdot 5H_2O$ in 1% potassium tartrate. After 15 mins, 0.5 ml of 1N Folin-phenol reagent (Fisher Scientific Company Limited) was added to each sample. After 30 min., the intensity of the blue color was determined at 630 mu using a Hitachi Perkin-Elmer spectrophotometer, model 139. The procedure was repeated and the average value for each point was used to prepare the standard curve (Fig. 1).

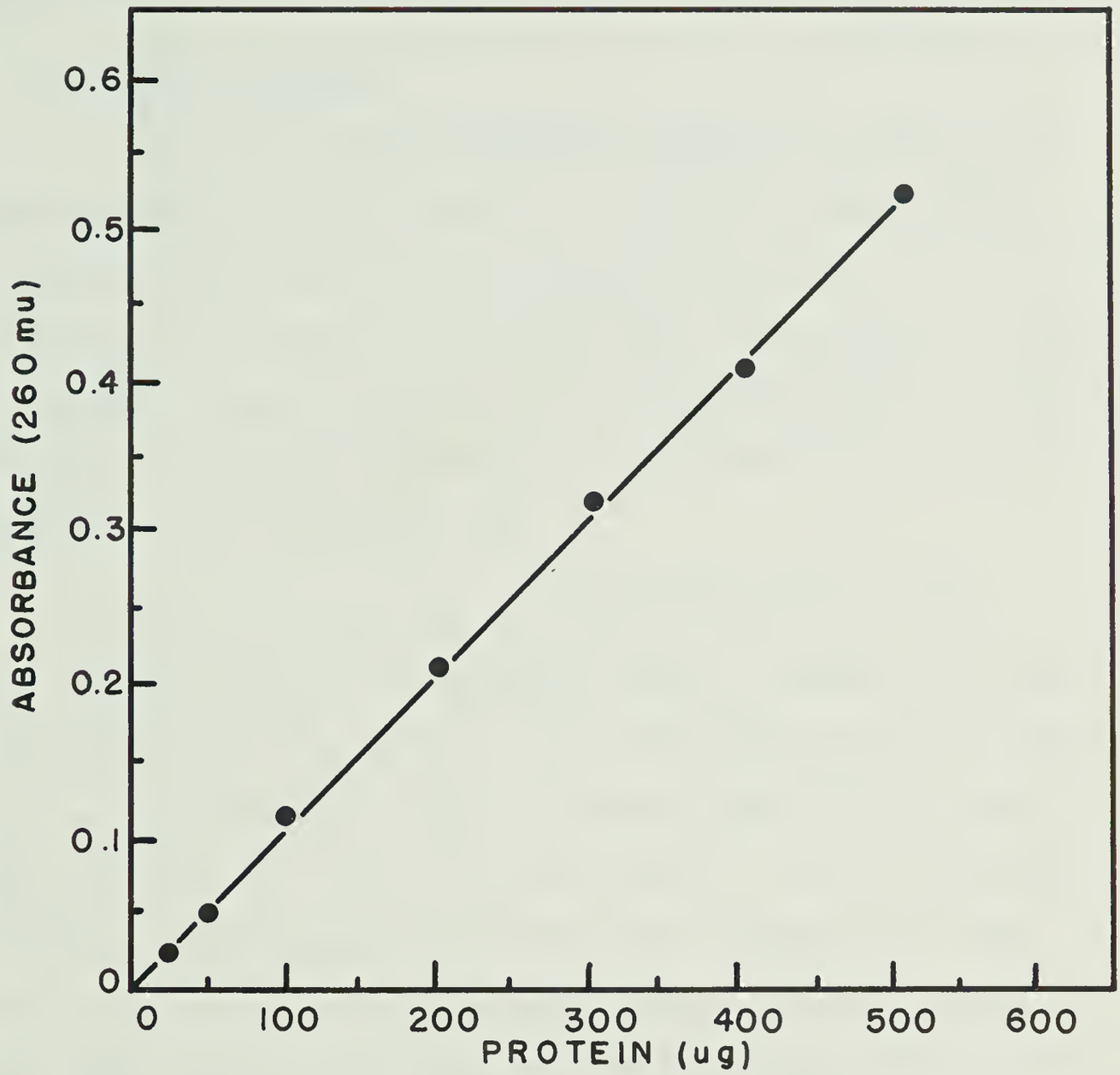


Fig. 1. Standard curve for protein determination.

D. DISC GEL ELECTROPHORESIS STUDIES

Disc gel electrophoresis was carried out for the separation of the soluble proteins and polyphenol oxidase and peroxidase isozymes.

(a) Soluble Proteins

The soluble proteins previously extracted (see above) were separated by disc gel electrophoresis as described by Davis (1964). An aliquot of the protein sample containing an equivalent of 300 ug of protein, as determined by the Lowry et al. (1951) method, was added to each gel. A constant current of 3 mA per gel was applied until the tracer dye (bromophenol blue) moved to a point marked 1.5 cm from the bottom of a tube containing the gel.

The gels were removed from the tubes with the aid of a jet of water running through a thin hypodermic needle. Proteins in the gels were stained with 1% amido black 10-B in 7% acetic acid for 60 minutes and the excess stain in the gels was removed using 7% acetic acid. Three replicate gels were used for each sample. The above extraction and disc gel electrophoretic procedures were repeated once. Since there was considerable variation in the uptake of the protein stain by individual protein bands, it was considered necessary to indicate the relative amounts of stain uptake by the proteins. Solid lines represent large amounts of protein and dotted areas very little.

The above electrophoretic studies of soluble proteins for mycelium and sclerotia were carried out concomitantly. However, during the later portion of this study it was found that sclerotia from the liquid malt-yeast medium did not produce apothecia. In order to follow the changes in proteins in all stages of growth and development, it was

necessary to change to a medium which produced sclerotia which in turn would produce apothecia. For this the synthetic medium described earlier was employed, since preliminary experiments indicated that this medium was satisfactory for this purpose. Sclerotia of S. sclerotiorum were harvested from 10-, 20-, 30-, 40-, 50-, 60-, and 70-day old cultures grown on the synthetic medium at 15°C. Apothecia were produced from sclerotia placed into H_2O as previously described. Extraction and electrophoretic procedures described above were used for the separation of soluble proteins, and peroxidase and polyphenol oxidase isozymes of the sclerotia from the above-aged cultures, sclerotia separated from the apothecia, and the apothecia.

(b) Comparative studies of S. sclerotiorum and Sb_3 and Sb_4

Using the above procedures, a comparative study of the proteins of sclerotia and apothecia of S. sclerotiorum and isolates Sb_3 and Sb_4 was made. The sclerotia of Sb_3 and Sb_4 were removed from 165-day old cultures prior to placing them in sterile, distilled and deionized H_2O.

Although sclerotia of S. sclerotiorum and Sb_3 and Sb_4, harvested for analyses differed considerably in their chronological age, the stage at which sclerotia produced apothecia indicated a somewhat similar physiological age.

(c) Peroxidase and polyphenol oxidase isozymes

Peroxidase and polyphenol oxidase isozymes were determined using disc gel electrophoresis on these samples used for protein determinations.

Peroxidases were detected with the methods involving benzidine-2HCl reagent (Bulletin--special subject, Enzyme Analysis, Canal Industrial Corporation, Rockville, Maryland) and pyrogallol (Hall 1967, Macko et al. 1967). Polyphenol oxidase isozymes were detected using 3.4-dihydroxphenylalanine (l-DOPA) (Macko et al. 1967) in 0.05 M phosphate buffer, pH 6.0.

E. AMINO ACID ANALYSES OF SCLEROTIA AND APOTHECIA

It appears that sclerotia of S. sclerotiorum require a maturation period (Purdy 1956). Since there were only minor differences in protein patterns (see Fig. 15), the possibility that this maturation period involved changes in amino acids was considered. Therefore analyses for the readily detectable free and bound amino acids were carried out on sclerotia and apothecia harvested at the same time as those used for protein analyses from cultures grown on the synthetic medium. The procedure used for extraction of free and bound amino acids is as follows.

(a) Extraction of amino acids

Sclerotia removed from the cultures were ground in the Krups grinder and 2 g of this material was suspended in 3 ml H_2O. This mixture was frozen at -48°C for 24 hours and then passed through a chilled Hughe's press. Sufficient 95% ethanol was added to the disrupted cells to give a final concentration of 80% alcohol, and additional 80% ethanol was added to give a final volume of 50 ml. After stirring for one hour, the solid particles were allowed to settle and the supernatant was decanted. Fifty ml of 80% ethanol was added to the precipitate and the stirring and decanting procedures were repeated. A final volume of 50 ml of 80% ethanol was added to the precipitate and the

mixture was stirred overnight. The solid and liquid portions were separated by filtration using a Whatman #2 filter paper. The above supernatant fractions were combined and filtered. The filtrate was evaporated to dryness at 40°C using a flash evaporator. The residue was taken up in 2 ml 10% isopropanol, and this fraction was used for free amino acid determinations. The alcohol insoluble fraction was removed from the filter paper and placed in small ampules to which was added 20 ml 6 N constant boiling HCl. The ampules were evacuated and sealed. These samples were hydrolysed by autoclaving for 24 hours at 121°C. After cooling, the samples were filtered using Whatman #2 filter paper. This residue was washed with 100 ml 80% ethanol and again filtered. The filtrates were combined and evaporated to dryness at 50°C using a flash evaporator to remove the HCl. The resulting residue was dissolved in 80% ethanol and again evaporated to dryness. The dissolving in ethanol and evaporation were repeated. The final residue was taken up in 2 ml 10% isopropanol. The pH of the bound amino acid sample was adjusted to 6.0 with N KOH, and the resulting precipitate removed by filtration with Whatman #2 filter paper was discarded.

Apothecia were also analysed for free and bound amino acids using the above procedures, except that the apothecia were not ground in the Krups grinder.

Free and bound amino acids were analysed using thin layar chromatography (TLC) ascending method, as described by Pataki (1966).

(b) Preparation of the TLC plates

In this procedure the TLC plates were prepared using silica gel G (Brinkmann Instruments Limited). A 20 g sample of silica gel G was

suspended in 40 ml H_2O, and this amount was sufficient to give a gel thickness of 0.25 mm covering five 20 x 20 cm glass plates. The plates were dried and activated at 105°C in a forced-air oven for 30 minutes. They were stored until required in an air-tight cabinet containing $CaSO_4$ as a desiccant.

(c) Development of the TLC plates

An aliquot of an amino acid sample was spotted in the lower right hand corner, 2 cm from either edge, of an activated TLC plate. Since the amino acid content of the extracts varied, aliquots ranging from 5-30 ul were used. The TLC plates were developed in two directions. The first solvent, which was butanol: acetic acid: H_2O (4:1:1), was allowed to run until the solvent front had travelled to within 3 cm of the top edge of the plate. The plates were air-dried thoroughly at room temperature using forced air. In the second direction, perpendicular to the first, the solvent used was 80% phenol (pH 5.5) containing 20 mg NaCN per 100 ml solvent. The phenol was prepared by heating 80 g of reagent grade phenol (Mallinckrodt Chemical Works) with 20 ml H_2O. The TLC plates were thoroughly air-dried as before and then sprayed with 0.3% ninhydrin in 100 ml butanol containing 3 ml acetic acid. The plates were then heated to 110°C for 10 minutes to develop the amino acid-ninhydrin color complex. The R_f values for each spot were compared to those of amino acids in a standard solution. This solution, containing the known amino acids, was prepared by adding 1 mg of a known amino acid per ml of 10% isopropanol. The amino acids of this standard solution were separated and identified on the TLC plates using the above solvent systems. The R_f value for each known amino acid was determined.

Two TLC plates were used for each amino acid sample and the above extraction and chromatographic procedures were repeated once.

F. FACTORS AFFECTING APOTHECIA PRODUCTION

In this study and those of Young (1934, 1936), Purdy (1956), Bedi (1962), and Williams and Western (1965) it was found that the production of apothecia of S. sclerotiorum was affected by substrate. Also apothecia production was increased when sclerotia were placed in soil treated with 1,3-dichloropropene, a fumigant used for nematode control (Paktyka and Mai 1958). Sproston and Pease (1957) found that an alternating temperature was required during the development of sclerotia of S. trifoliorum for successful production of apothecia. The following studies were made to determine the effects of some compounds and temperature on apothecia production of S. sclerotiorum.

(a) Plant extracts

A study was made to determine if plant extracts would affect apothecia production of S. sclerotiorum. However, the stage at which the plant extracts could inhibit or promote apothecia production is not known. That is, would the extracts have a greater effect on apothecia production if they were present during the growth of the sclerotia, or is the effect greater on mature sclerotia?

(i) Sclerotia grown on the synthetic-agar medium plus plant extracts. Sclerotia were grown on the synthetic medium to which was added aqueous extracts of mature barley roots and aboveground parts respectively, mature rape roots and aboveground parts respectively, and immature sunflower aboveground parts. The control was the synthetic medium only.

For preparing the plant extracts, the roots and aboveground parts of the plants were separated, air-dried for 24 hours, and oven-dried at

60°C in a forced air oven for 24 hours. Each was ground in a Wiley mill using a 60 mesh screen. Three grams of the resulting powder were extracted with 200 ml of H_2O. The extraction was carried out by constantly stirring the powder-H_2O mixture for 4 hours at room temperature. The extract was separated from the cellular fraction by filtration through a Whatman #2 filter paper and then was sterilized by filtration using a 0.22 mu millipore filter. A portion of this filtrate was added to the warm and still liquid agar-synthetic medium (1 : 1) and sterilized by autoclaving. For this study the synthetic medium was prepared at 2 x the normal concentration so that when diluted with the plant extracts, the normal concentration of synthetic medium would be obtained. Cultures of S. sclerotiorum were grown on this medium at 15°C. The sclerotia from 70-day old cultures were removed and placed into sterile H_2O and kept at 15°C under artificial illumination (see page 5) for 30 days.

(ii) Mature sclerotia germinated in the plant extracts. Sclerotia from 70-day old cultures, grown on the synthetic medium at 15°C, were placed in a series of petri plates containing 20 ml aliquots of plants extracts for 30 days. A portion of the extracts were diluted with H_2O so that the ratios of extracts to H_2O were 1:1, 1:2, 1:9, and 1:99. Controls included sclerotia placed in undiluted extracts and H_2O respectively.

(b) Glucose concentration

Sclerotia from cultures grown at 15°C on a malt-yeast agar medium containing 0, 10, 20, and 30 g glucose per liter were placed into sterile H_2O and incubated at 15°C in the light.

(c) 1,3-dichloropropene

Sclerotia taken from 70-day old cultures grown on the synthetic medium were soaked in a 0.1% 1,3-dichloropropene solution for 3 days at 15°C. The concentration of 0.1% 1,3-dichloropropene was extrapolated from data given by Paptyka and Mai (1958) who used this chemical in their fumigation studies. The sclerotia were placed into sterile H_2O and incubated at 15°C in the light. The controls were sclerotia soaked in H_2O.

(d) Effects of light and dark

In experiments reported by Henderson (1962) and Bedi (1962), it was found that sclerotia incubated in the dark produced long thread-like initials, but apothecial heads (i.e. apothecia with asci and ascospores) were not formed. However, if sclerotia were incubated in the light, normal saucer-shaped apothecia were formed. Therefore a comparative study was carried out to determine the effects of light and dark treatments on the ability of sclerotia to produce mature apothecia.

-Chemical treatments. It has been found that various compounds could be used to replace light in fructification of certain fungi (Zafar and Colotelo 1969, and Khan 1966). Using Zafar and Colotelo's approach, horseradish peroxidase (Nutr. Biochem. Corp.), H_2O_2, peroxidase plus H_2O_2, indole acetic acid (IAA), polyphenol oxidase (Nutr. Biochem. Corp.), and fungal catalase (Nutr. Biochem. Corp.), respectively were used to determine whether these compounds could substitute for light in the production of apothecia of S. sclerotiorum. The following concentrations of the compounds were used: peroxidase (activity- 10 purpurogallin units per ml), H_2O_2 (0.1%), peroxidase (activity- 10 purpurogallin units per ml) plus H_2O_2 (0.1%), IAA (25

and 50 ppm respectively), polyphenol oxidase (10 units/ml), and fungal catalase (10 units/ml). Sclerotia taken from 70-day old cultures, grown on a synthetic medium were placed into petri plates containing 20 ml of each of the above solutions and kept in the light and dark at 15°C for 30 days. The controls contained only H_2O. The dark experiments using various chemicals were repeated once.

H. PIGMENTATION OF SCLEROTIA

In the development of sclerotia, young sclerotia become pigmented, light green, and in a short period of time, e.g. 2 days, are black. A study was made to determine the effects of various compounds on the growth and pigmentation of sclerotia. The compounds selected for this study were ones which have been reported to affect fructification by various means e.g. enzyme inhibition and melanin synthesis. The compounds used, references as to their use, and purpose used are shown in Table I.

The compounds and concentrations used are as follows: phenylthiourea (10^{-3}M); sodium diethyldithiocarbamate (dieca) (10^{-4} M, 2×10^{-4} M, 10^{-3} M, 2×10^{-3} M, and 5×10^{-3} M); cysteine (10^{-3} M and 10^{-2} M); thiouracil (10^{-3} M); thiourea (10^{-3} M); dimethylsulfoxide (10^{-3} M); potassium cyanide (10^{-3} M); disodium ethylenediaminetetraacetate (Na_2-EDTA) (10^{-3} M); peroxidase (activity- 10 purpurogallin units/ml); polyphenol oxidase (activity- 10 units/ml); catalase (activity- 10 units/ml); iodoacetic acid (10^{-5} M and 10^{-4} M); and iodoacetamide (10^{-5} M and 10^{-4} M). In the preparation of these solutions, the stock solution (2 x concentration desired) was sterilized using a 0.22 mu millipore filter and diluted with an equal volume of sterile (autoclaved), liquid

COMPOUND	REFERENCE	PURPOSE USED
sodium diethyldithiocarbamate	James 1953	enzyme inhibition
	Bonner 1957	enzyme inhibition
	Carley et al. 1967	pigment inhibition
phenylthiourea	Lerner & Fitzpatrick 1950	melanin inhibition
	Bonner 1957	enzyme inhibition
	Hirsch 1959	melanin inhibition
cysteine	Lerner & Fitzpatrick 1950	melanin inhibition
	Hirsch 1959	melanin inhibition
	Chet et al. 1966	inhibition of growth
thiouracil	Lerner & Fitzpatrick 1950	melanin inhibition
	Bonner 1957	enzyme inhibition
dimethlysulfoxide	Carley et al. 1967	pigment inhibition
potassium cyanide	Hirsch 1957	melanin inhibition
	Carley et al. 1967	pigment inhibition
disodium ethylenediaminetetraacetate	Hirsch 1957	melanin inhibition
	Urey & Horowitz 1967	inhibition of tyrosinase
	Chet & Henis 1968	induction of sclerotia
peroxidase	MacMillan & Brandt 1966	melanin synthesis
polyphenol oxidase	Lerner & Fitzpatrick 1950	melanin synthesis
	Chen & Chavin 1965	melanin synthesis
catalase	Saunders et al. 1964	substitute for peroxidase
iodoacetic acid	James 1953	enzyme inhibition
	Chet et al. 1966	induction of sclerotia
	Chet & Henis 1968	induction of sclerotia
iodoacetamide	James 1953	enzyme inhibition
	Chet & Henis 1968	induction of sclerotia

Table I. Compounds used in pigmentation and growth studies, references as to their use, and purpose used.

malt-yeast medium. A 40-ml aliquot of this medium was added per petri plate. A single sclerotium was placed in each petri plate and incubated at 20°C for 20 days. The mycelium and sclerotia were separated and the dry weights were taken as previously outlined. Two petri plates were used for each treatment and the experiment was repeated once.

RESULTS AND DISCUSSION

A. GROWTH STUDIES

Stages in the development of sclerotia are shown in Fig. 2 (a-e). Four days after inoculation the mycelial colony on the liquid malt-yeast medium was almost the full diameter of the petri plate (Fig. 2a). On the fifth day, small, white developing sclerotia were observed (Fig. 2b). At this stage small clear liquid droplets could be seen on the surface of the sclerotia. This liquid has been observed from a number of sclerotia producing fungi by DeBary (1887), MacDonald (1934), Young (1934), Brodie (1935), Remsberg (1940), and Cooke (1969). Remsberg (1940) observed a crystalline substance when liquid from developing sclerotia of Typhula spp. was placed on a glass microslide and dried. The liquid from sclerotia of S. sclerotiorum and S. trifoliorum was found to contain carbohydrates (Cooke 1969). In unpublished data, Colotelo and Voegelin (1970) found this liquid to be of a very complex nature, containing proteins, enzymes, carbohydrates, amino acids, and inorganic ions. This liquid appears therefore to be involved with rapid development of sclerotia. Cooke (1969) suggests that since sclerotia are resting structures and show a reduced level of hydration compared with vegetative organs, maturation must therefore involve some water loss and one obvious method is active excretion through the sclerotium surface, where it can evaporate or leak away. Within a short period of time (2-3 days), these droplets increased greatly in size and coalesced, but on mature sclerotia no liquid was observed. This phenomema was also noted by Cooke (1969). MacDonald (1934) also observed that the exudates present on developing sclerotia of Typhula gyrans

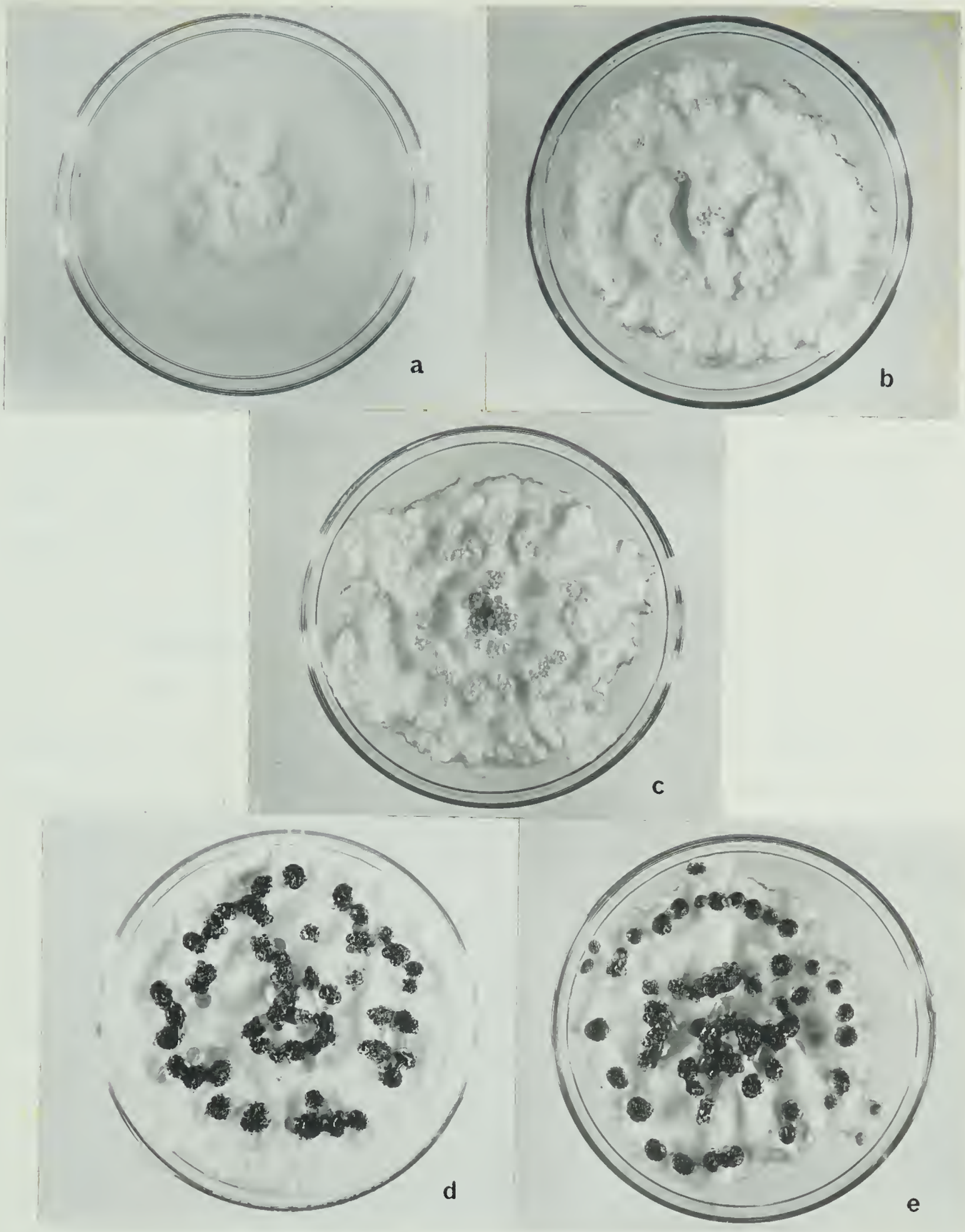


Fig. 2 (a,b,c,d,e). Cultures of S. sclerotiorum in various stages of growth on a liquid malt-yeast medium at 15°C. (a) 4-day old cultures, (b) 5-day old cultures, (c) 6-day old cultures, (d) 7-day old cultures, and (e) 8-day old cultures.

were not present on mature sclerotia. Six days after inoculation the sclerotia had enlarged considerably and were starting to turn green in color (Fig. 2c), and on the seventh day they were green-black (Fig. 2d). Sclerotia harvested 8 days after inoculation were completely black (Fig. 2e). In Sclerotium rolfsii the pigment extracted from sclerotia was found to be melanin-like in nature (Chet et al. 1967). Results for studies on pigmentation of sclerotia of S. sclerotiorum are presented in a later section (page 59). It appeared that sclerotia had reached their maximum size by the fifteenth day, after which the sclerotia were probably in a maturation period. This is supported by the following data.

(a) Fresh and dry weight of mycelium and sclerotia

The fresh and dry weights for mycelium and sclerotia, on a colony basis and percent dry weight basis, harvested at various times after inoculation, are shown in Fig. 3. Each point in Fig. 3 is the average of 4 weights.

On a colony basis (Fig. 3), peaks for fresh and dry weight of mycelium were obtained at 5 and 9 days. The fresh and dry weights of mycelium were highest at the stages when the sclerotia were rapidly increasing in weight and size. After 9 days, the fresh weight of mycelium decreased rapidly, and from 20-30 days the level was fairly constant. A constant level for fresh weight of mycelium was obtained after 20 days, but a constant level for dry weight was obtained after 10 days. Beginning on the fifth day, the fresh and dry weight of sclerotia increased rapidly and the weights were fairly constant after 15 days (Fig. 3). The fresh and dry weights of sclerotia from one colony were

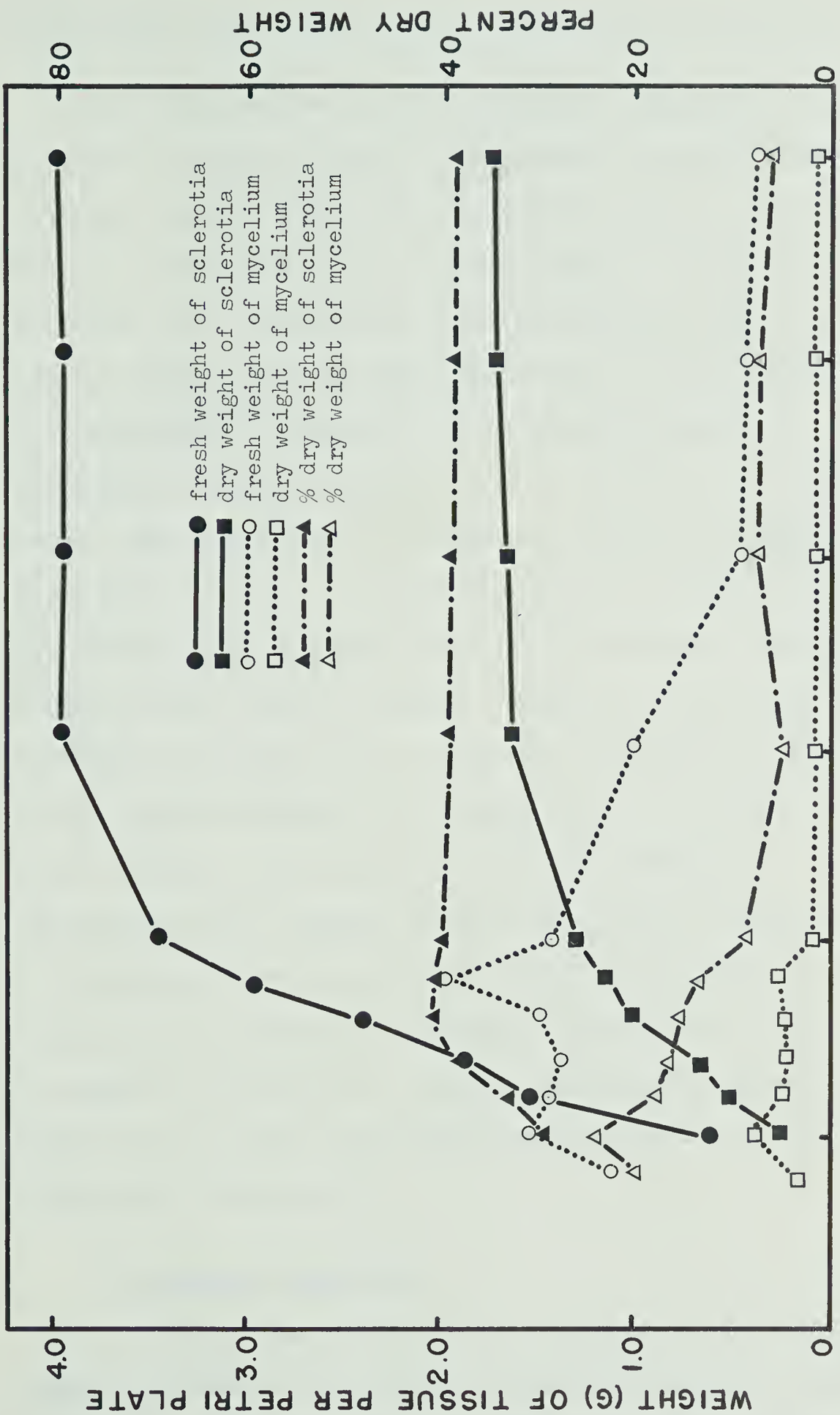
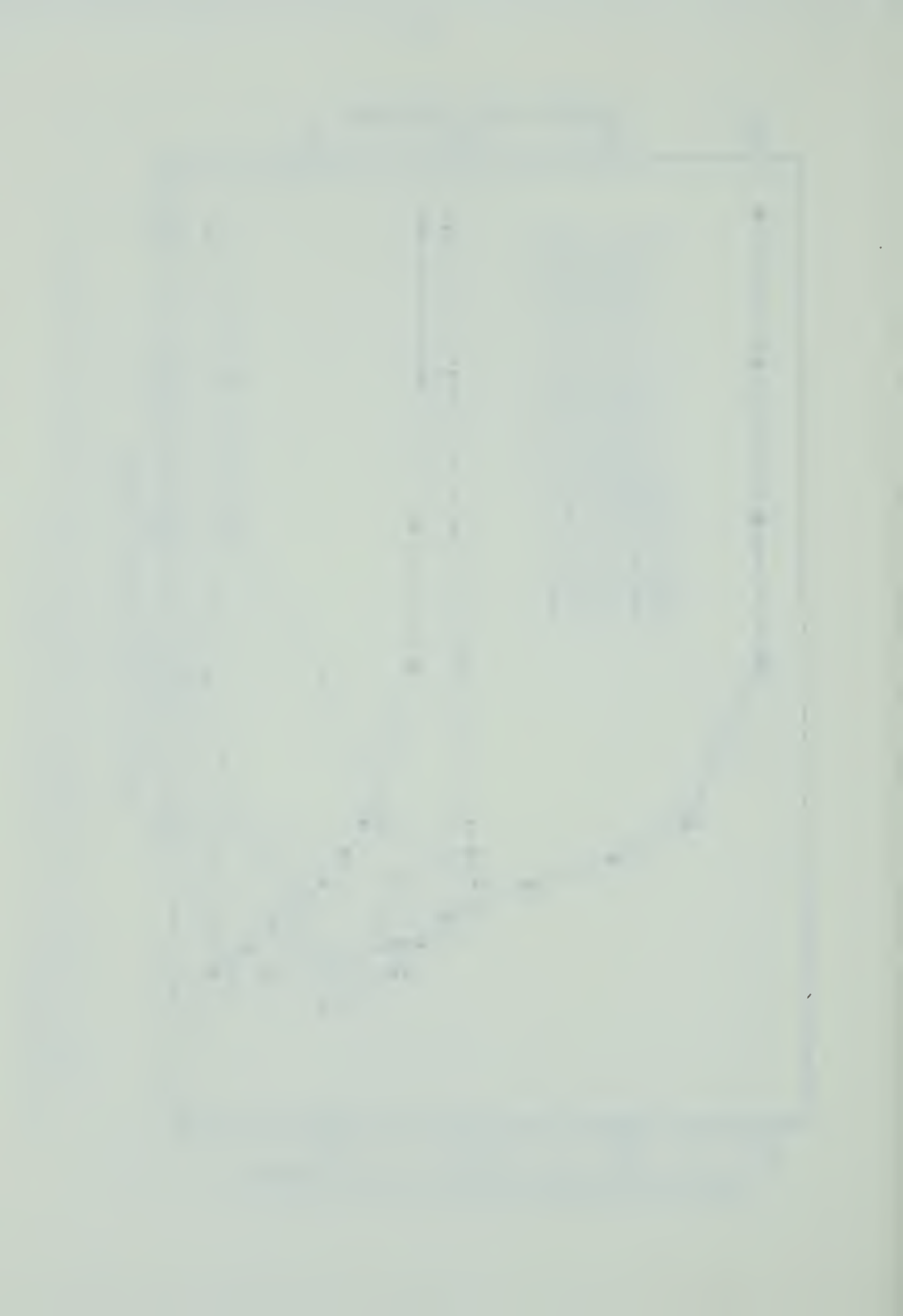


Fig. 3. Fresh and dry weights, and percent dry weights of mycelium and sclerotia of S. sclerotiorum grown on a liquid malt-yeast medium at 20°C.



considerably higher than those of the mycelium of that colony.

On a percentage basis, the dry weight of sclerotia was much higher than that of mycelium (Fig. 3). The percent dry weight of mycelium increased until such time that the sclerotia began to develop rapidly. Then the percent dry weight decreased rapidly. The rapid decline may have been due to senescence in which the mycelium broke down and released compounds into the medium and/or due to translocation of chemical components of mycelium into the developing sclerotia. The use of radioactive tracers incorporated into the sclerotia would clarify this issue. Concomitant with the decrease in percent dry weight of mycelium, there was an increase in percent dry weight of sclerotia. Relative to age of cultures, the percent dry weight of sclerotia increased up to 8 days and then remained constant. However, the dry weight of sclerotia continued to increase until 15 days after inoculation. The increase in dry weight of sclerotia was possibly due to an increase in number of cells, cellular constituents, and cell wall thickening. A possible explanation of the increase in percent dry weight of sclerotia is that the liquid, seen on the surface of developing sclerotia and which contains cellular constituents (Colotelo and Voegelin 1970), could possibly be exuded by the sclerotia. Colotelo and Voegelin collected approximately 2.0 ml of this liquid from developing sclerotia from one large petri plate (138 mm i.d.).

(b) Apothecia production

For reasons stated earlier (page 5), cultures of *S. sclerotiorum* were grown on the synthetic medium for studies on apothecia production. Using the synthetic medium, the stages of sclerotial

development were similar to that obtained using the above liquid malt-yeast medium; however, growth was slightly slower. The production of black sclerotia took 2-3 days longer than from cultures grown on the liquid malt-yeast medium.

Sclerotia from 70-day old cultures kept in sterile H_2O for 30 days produced typical mature apothecia (Fig. 4). Some sclerotia produced as many as 7 apothecia per sclerotium, whereas others produced none. The percent germination of sclerotia of this isolate appears to be low as compared to the studies by Bedi (1962 and 1963). In his studies he obtained 100% germination of the sclerotia placed in H_2O and these sclerotia produced from 1 to 15 apothecia. Purdy (1956) found that some isolates of S. sclerotiorum form apothecia readily and rapidly, whereas sclerotia of other isolates form apothecia more slowly and sporadically. It would appear, therefore, that further nutritional and/or environmental studies on sclerotia of the isolate used in these studies would be in order to try to increase apothecia production.

Measurements of expanded apothecia (Fig. 4), and asci and ascospores (Fig. 5) of S. sclerotiorum are as follows: The asci were 8.2 u x 130 u and the ascospores were 4.4-5.0 u x 8.8 u. Paraphyses were present. The size of ascospores and asci of S. sclerotiorum are in agreement with the sizes reported by Ramsey (1925), Keay (1939), and Purdy (1955) for their isolates.

Sclerotia of Sb_3 and Sb_4 from 165-day old cultures grown on the malt-yeast-agar medium, kept in sterile H_2O for 75 days, produced mature apothecia (Figs. 6 and 8 respectively). Several asci containing asco-

Fig. 4. Apothecia of S. sclerotiorum still attached to sclerotium taken from 70-day old cultures, grown on the synthetic-agar medium at 15°C and germinated in H_2O. Magnification 5X.

Fig. 5. Asci containing ascospores, and paraphyses in crushed apothecia of S. sclerotiorum. Magnification 400X.

spores, as seen in the crushed apothecia (Figs. 7 and 9 respectively), were measured. The asci of Sb_3 measured 11.2-12.5 u x 222.5-237.5 u, and ascospores measured 7.8-8.1 u x 15 u. The asci of Sb_4 measured 11.2-12.0 u x 227.5-247.5 u and the ascospores measured 7.5-8.1 u x 14.4-16.3 u. Paraphyses were present in both isolates. The measurements for ascospores of Sb_3 and Sb_4 are slightly smaller than that given for S. borealis by Groves and Bowerman (1955) and Sprague et al. (1961). However, the size of the asci are in agreement with those given by these workers. From the measurement data, the Sb_3 isolate obtained from Smokey Lake, Alberta is very similar to the isolate (Sb_4) obtained from Beaverlodge, Alberta. It should be noted here that under natural conditions S. sclerotiorum produced apothecia in the spring and early summer (Ramsey 1925, Williams and Western 1965) and S. borealis produces apothecia in the very late fall (Sprague et al. 1961).

B. SOLUBLE PROTEINS

The results for total amounts of soluble proteins extracted from mycelium and sclerotia of a single colony are shown in Fig. 10. Results are also expressed on a dry weight basis (Fig. 11). As with the fresh and dry weights of mycelium (Fig. 3), two peaks for amounts of soluble protein per colony were obtained in 5-day and 9-day old cultures respectively. On a colony basis there was slightly more protein in the mycelium of 5-day old cultures as compared to those harvested at 9 days. The protein content of sclerotia, on a colony basis, increased rapidly and was fairly constant after 15 days and after 6 days was higher than that observed for mycelium.

The protein content of mycelium on a dry weight basis was higher

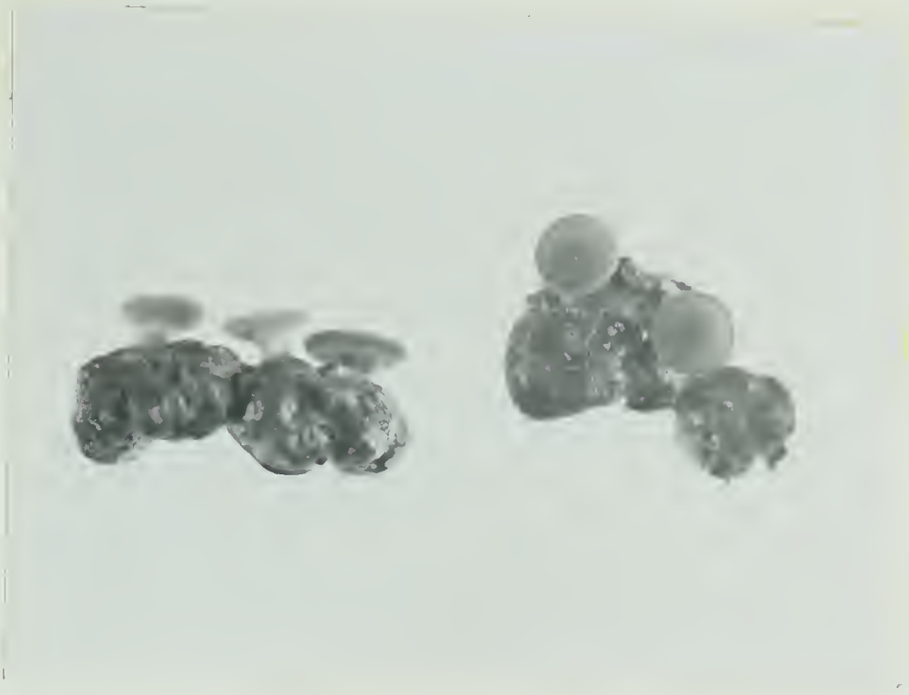


Fig. 6. Apothecia of S. borealis (Sb_3) produced by sclerotia taken from 165-day old cultures grown on a malt-yeast-agar medium at 10°C and germinated in H_2O. Magnification 2.5X.

Fig. 7. Asci containing ascospores, and paraphyses in crushed apothecia of S. borealis (Sb_3). Magnification 530X.

Fig. 8. Apothecia of S. borealis (Sb_4) produced by sclerotia taken from 165-day old cultures grown on a malt-yeast-agar medium at 10°C and germinated in H_2O. Magnification 2.5X.

Fig. 9. Asci containing ascospores, and paraphyses in crushed apothecia of S. borealis (Sb_4). Magnification 570X.

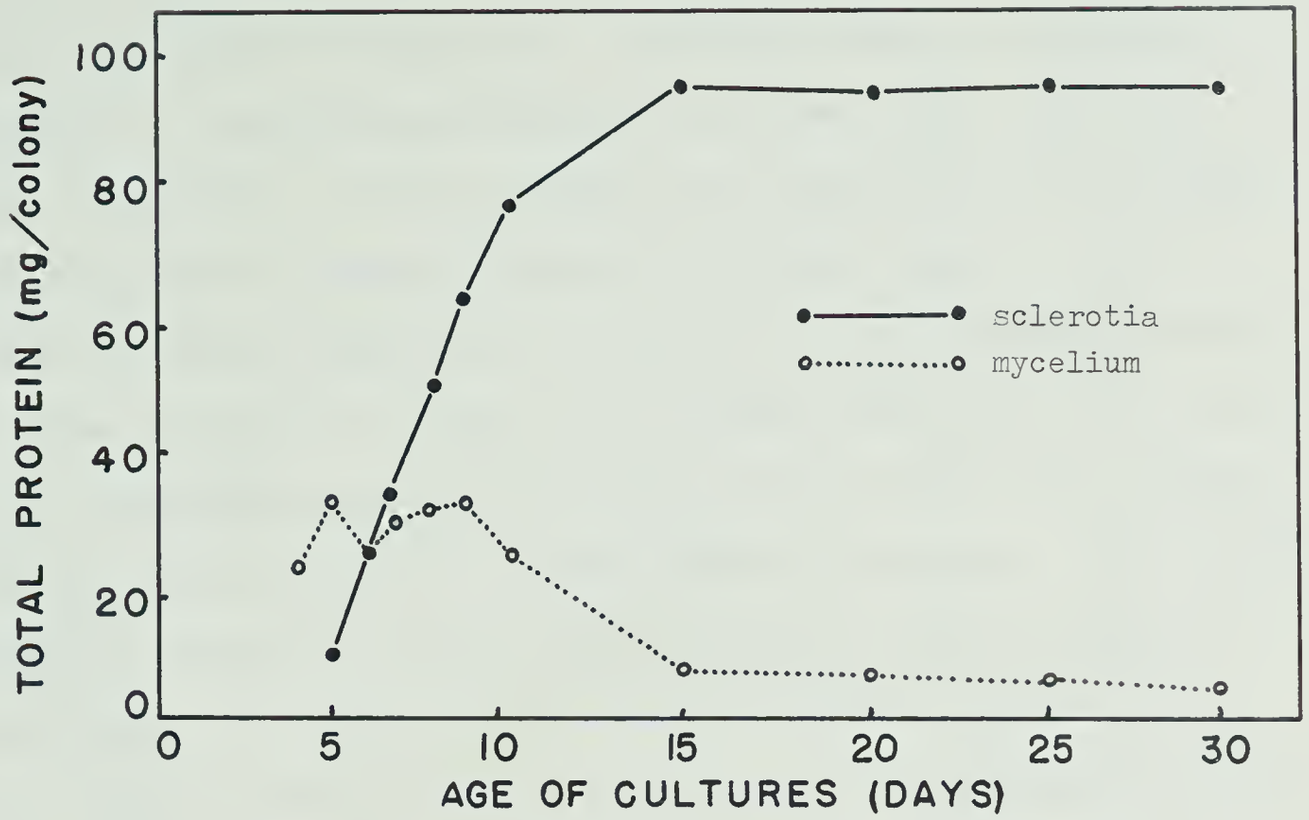


Fig. 10. Total soluble proteins (mg/colony) of mycelium and sclerotia from cultures of S. sclerotiorum grown on a liquid malt-yeast medium at 20°C.

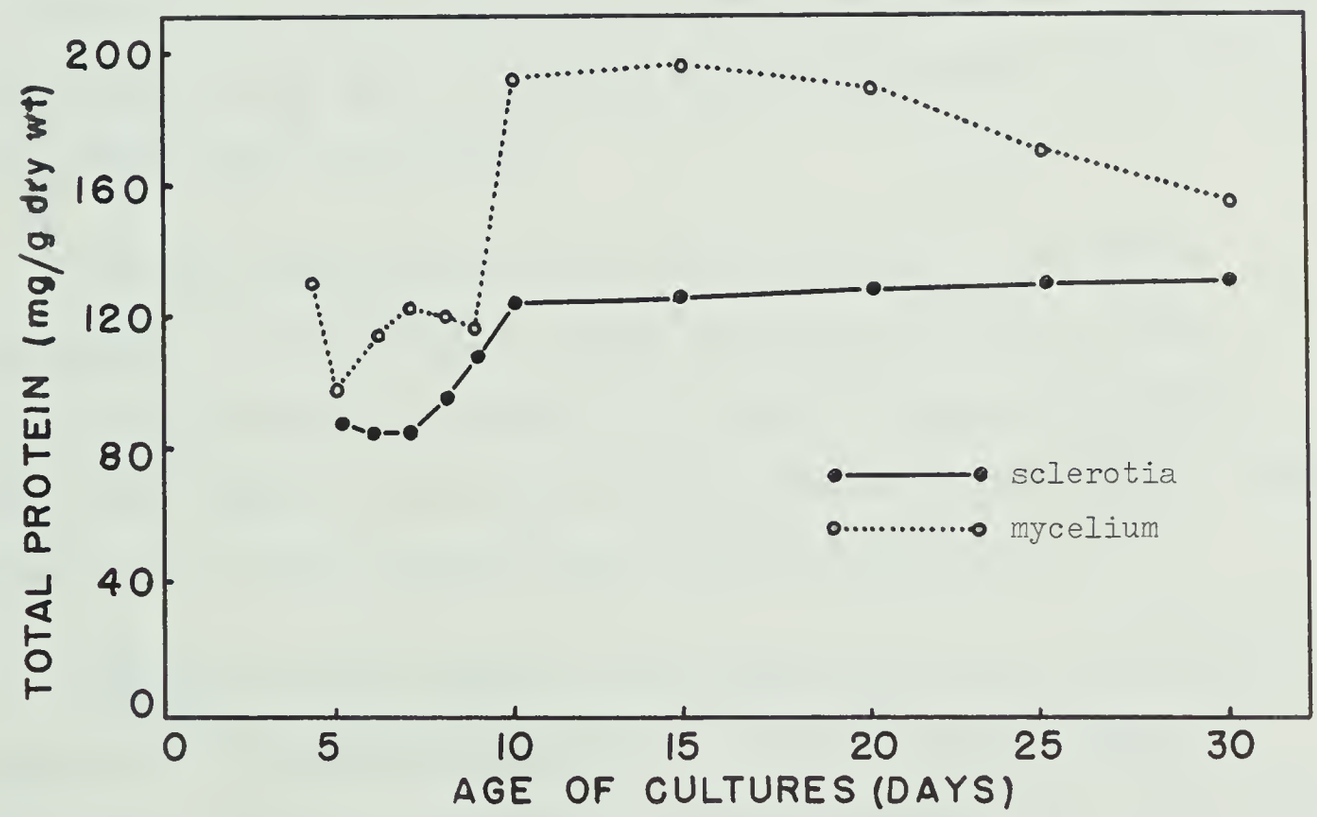


Fig. 11. Total soluble proteins (mg/g dry wt) of mycelium and sclerotia from cultures of S. sclerotiorum grown on a liquid malt-yeast medium at 20°C.

than that observed for sclerotia. A very high level of protein was obtained at 10 days and maintained for 10-20 days after which there was a slow decline. Prior to 10 days, the protein values for mycelium were reciprocal to those of mycelial dry weights (Fig. 3). The increase in protein of mycelium as expressed on a unit dry weight basis, as shown in Fig. 11, was rapid. This increase could result from a movement of non-proteinacious materials from the mycelium to the sclerotia with the protein content of the mycelium being unchanged. This is suggested by the drop in the dry weight of mycelium (Fig. 3) between 9 and 10 days. The total protein content in mycelium per colony shows that there was a drop in protein content during this same period but it was not proportional to the loss in dry weight of the mycelium. After this period (from 9-10 days) the mycelium probably began to senesce. On a dry weight basis maximum total soluble protein content of sclerotia was obtained in 10-day old cultures, and in 10- to 30-day old cultures the protein level was constant.

The only other data for total protein content of mycelium and sclerotia which is available, appears to be that of Chet and Henis (1966) for mycelial and sclerotial cell walls of Sclerotium rolfsii. They found that on a dry weight basis mycelial and sclerotial cell walls contained 2.7 and 2.6 percent total proteins respectively.

The total soluble proteins (mg/g fresh weight) of sclerotia from cultures of S. sclerotiorum grown on a liquid malt-yeast medium at 20°C and a synthetic-agar medium at 15°C were compared (Fig. 12). The protein content of the sclerotia, from cultures grown on the liquid malt-yeast medium, increased at a much faster rate than for sclerotia

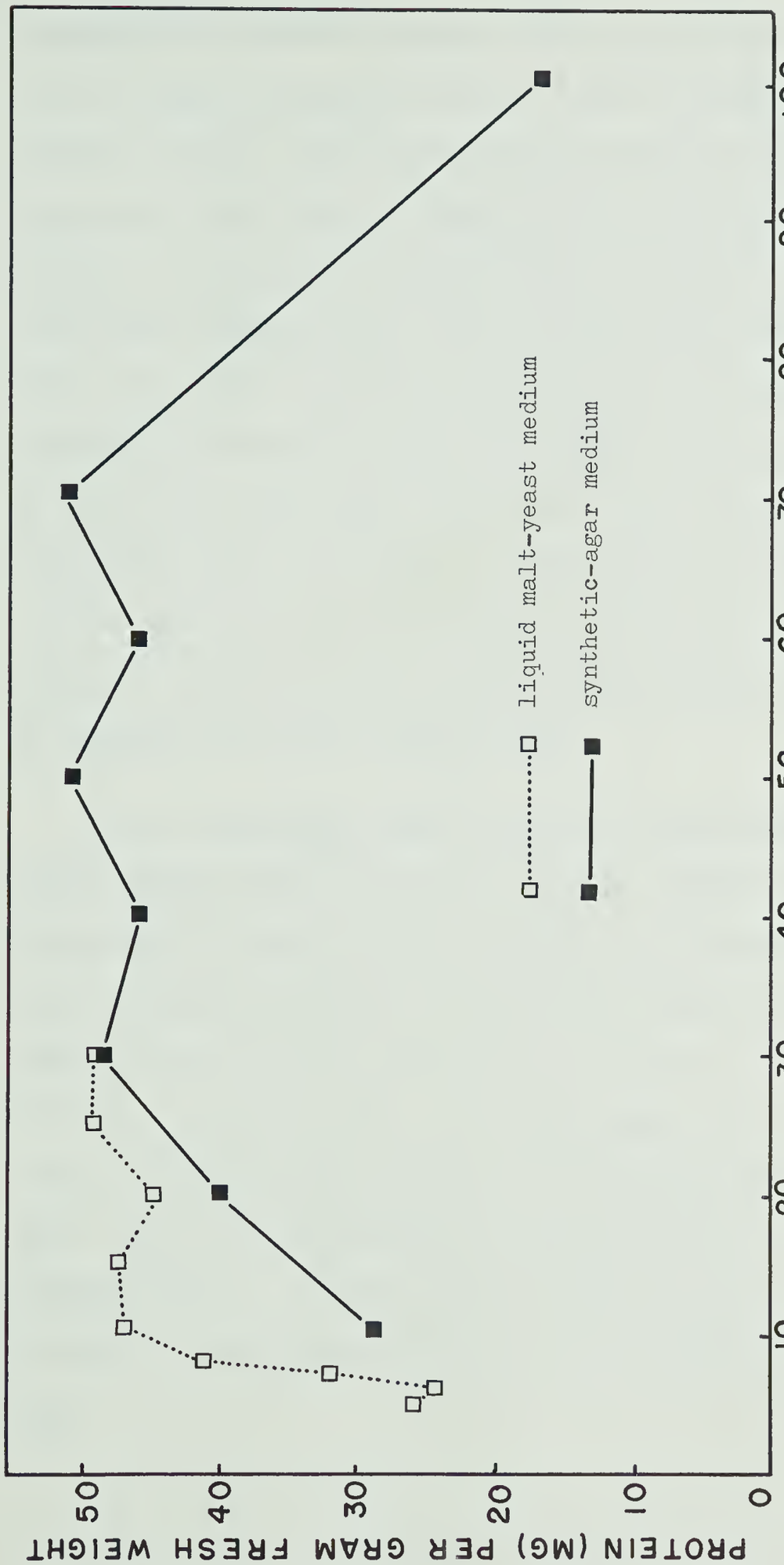


Fig. 12. Total soluble proteins (mg/g fresh wt) of sclerotia from cultures of S. sclerotiorum grown on a liquid malt-yeast medium at 20°C and a synthetic medium at 15°C.

cultured on the synthetic medium. This was probably due to the fact that the liquid malt-yeast medium is a more 'complete' medium than the synthetic medium. The liquid malt-yeast medium contains more nutrients and growth factors than are present in the synthetic-agar medium. The decrease in protein of sclerotia taken from cultures grown on the synthetic-agar medium between 70 and 100 days was probably due to an uptake of H_2O and/or a movement of proteins from the sclerotia to the apothecia. The uptake of H_2O is considered since sclerotia were placed in H_2O for the production of apothecia. One of the discrepancies in this study is that comparisons are made between sclerotia produced by cultures grown on the liquid malt-yeast medium and the solid synthetic medium. On a solid medium less nutrients would be available as compared to cultures grown on the liquid medium.

In the Lowry et al. (1951) method for total protein determinations many substances such as uric acid, guanine, xanthine, phenols (except nitrophenols), ammonium sulfate, tyrosine, and tryptophan increase color intensity and glycine decreases color intensity. Using this procedure, sucrose has also been found to interfere with color development (Gerhardy and Beevers 1968). The color varies with different proteins and is not directly proportional to protein concentration (Lowry et al. 1951). With the concentrations of proteins between 10 and 500 mg, the standard curve is a straight line in which the color intensity is proportional to concentration and the error is, therefore, kept to a minimum.

C. DISC GEL ELECTROPHORESIS

(a) Soluble proteins

In general, the number and the electrophoretic patterns of protein bands of buffer soluble proteins extracted from mycelium harvested at various times were very similar but in the 30-day old cultures there was a marked reduction in numbers of bands (Fig. 13). Except in mycelium of 4-, and 25-day old colonies, there were three prominent protein bands. The remaining bands were faint in outline since they had not taken up the amido black to the same extent as the prominent bands. The maximum number of bands occurred in 5-day old colonies at which time sclerotia were first evident. The changes in numbers of bands occurred in the lower thirds of the gels with the number of bands in the center portion remaining fairly constant. There were significantly fewer bands, only 8, in mycelium harvested from 30-day old cultures. From the mycelium of 30-day old cultures, no protein bands were observed in the lower third of the gel. As well, there was a decrease in numbers of bands in the upper third of the gel as compared to mycelium harvested at earlier dates.

The total protein content of mycelium, as determined by the Lowry et al. (1951) method, decreased rapidly after 10 days (Fig. 10). However, this decrease was not reflected in a reduction in number of protein bands, obtained on the polyacrylamide gels, until the cultures were 30 days old. Between 10 and 15 days after inoculation, when the greatest reduction in total proteins per colony occurred, there were very few changes in protein patterns, except for the loss of two bands in the lower region of the gels for 15-day old cultures. This loss may be

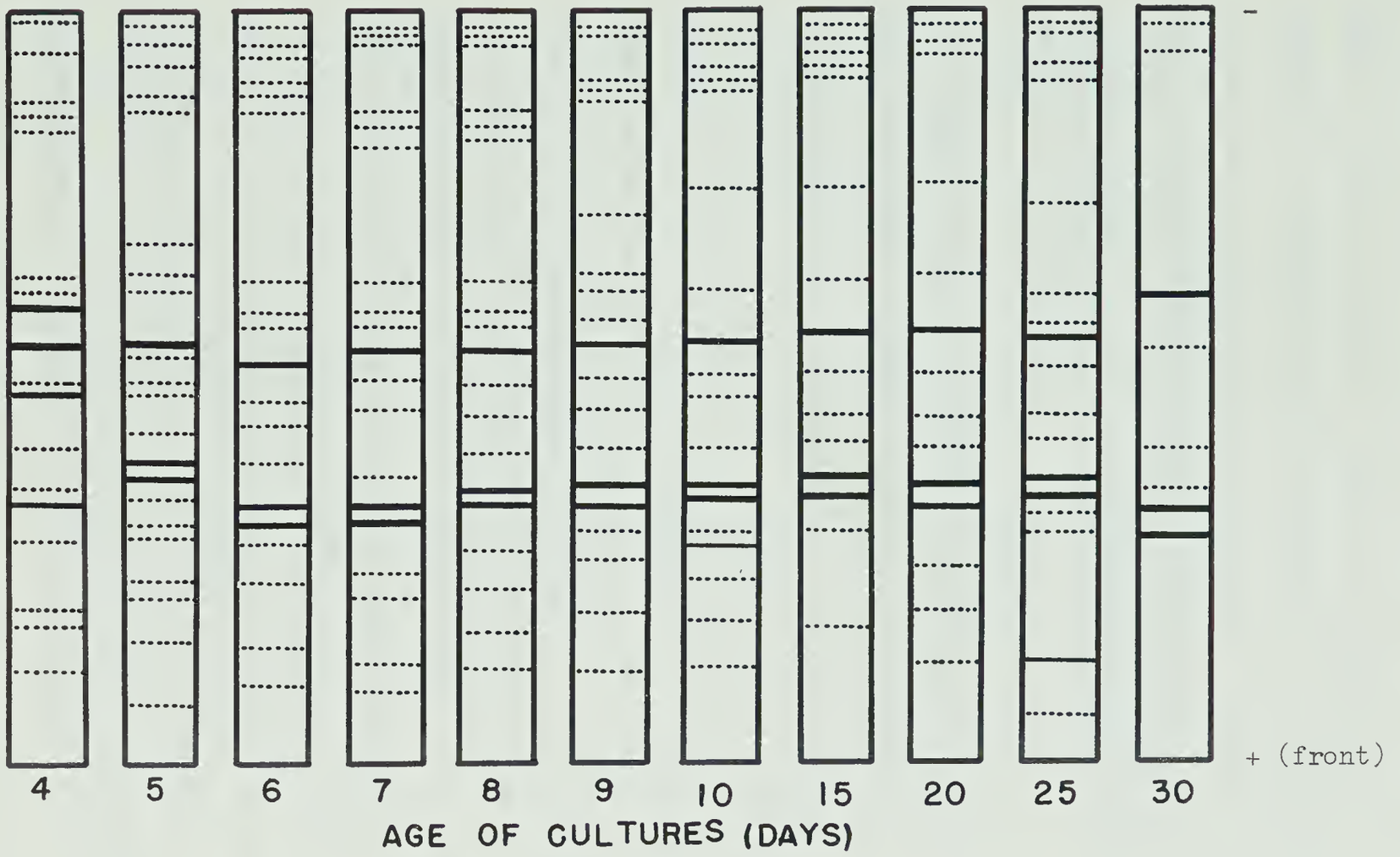


Fig. 13. Disc gel electrophoretic patterns of soluble proteins of mycelium of S. sclerotiorum from cultures grown on a liquid malt-yeast medium at 20°C.

responsible for the decrease in total protein values between 10 and 15 days as shown in Fig. 10. This also indicates that if there was a movement of proteins into the sclerotia, or if there was a decomposition of proteins, it was such that the ratio of different proteins in the mycelium remained fairly constant. The decrease in number of bands of soluble proteins from mycelium from 30-day old cultures indicates senescence of the mycelium.

Fig. 14 shows the patterns of soluble proteins of sclerotia removed from those cultures from which the mycelium was analyzed for proteins by disc gel electrophoresis. The most noticeable feature of protein patterns for sclerotial extracts is that there were three similar prominent bands present in the gels throughout the study. Of these three bands, two are wide and heavily stained with amido black. The major bands of sclerotia were identical to those of mycelium; however, in mycelium these bands were very narrow but in sclerotia two bands increase in width very markedly after 5 days. For older sclerotia i.e. those harvested from 20-, 25-, and 30-day old cultures, there was an increase in heavily stained bands occurring in the lower portion of the gels. The largest number of bands was observed for sclerotia harvested from 6-day old cultures at which time the developing sclerotia had started to turn green in color. After 5 days, the numbers and patterns of proteins were constant in the center portion of the gels.

The protein patterns obtained from extracts of sclerotia from colonies grown on the synthetic medium at 15°C are shown in Fig. 15. There were fewer bands present in extracts from sclerotia cultured on the synthetic medium at 15°C than from sclerotia cultured on the liquid

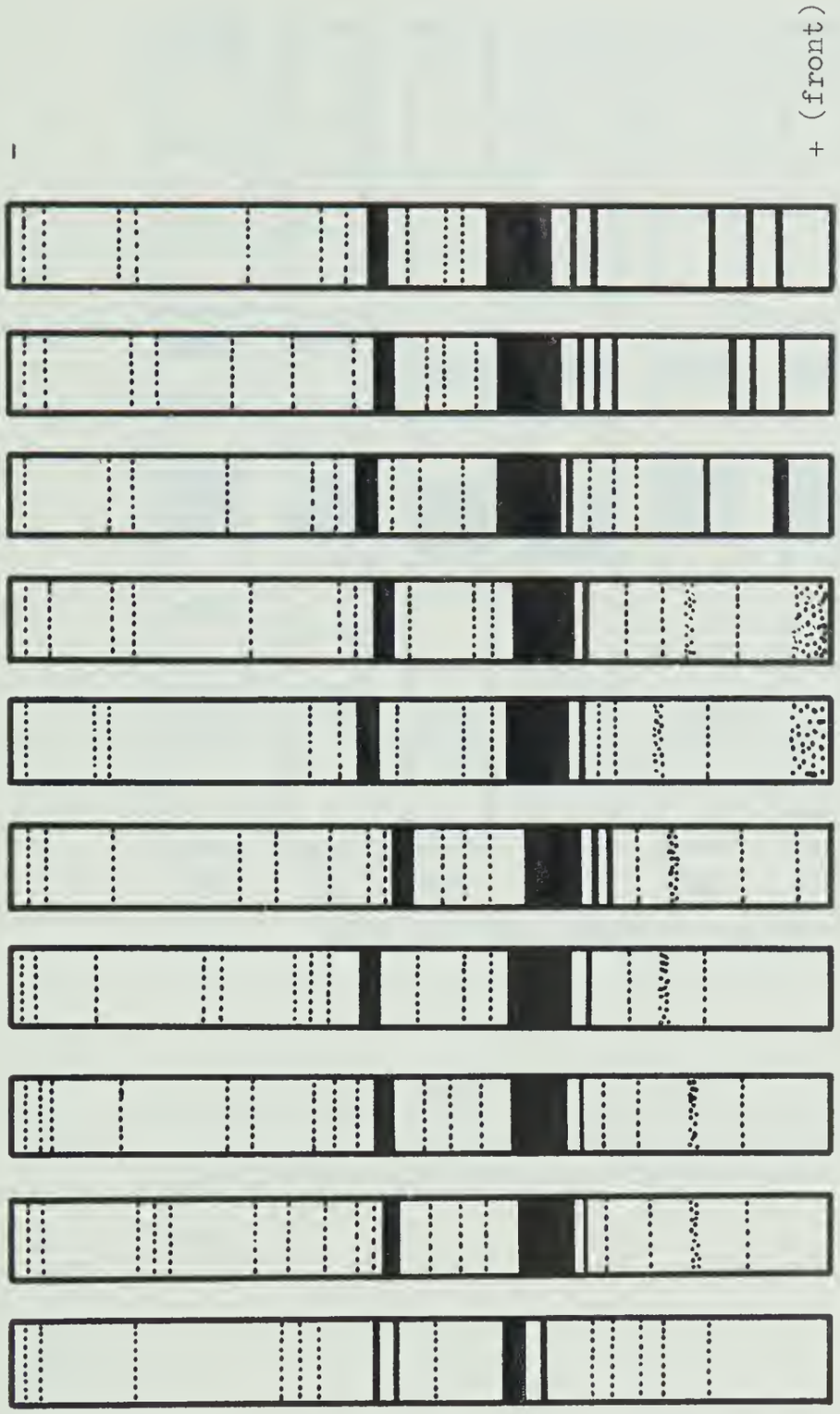


Fig. 14. Disc gel electrophoretic patterns of soluble proteins of sclerotia of S. sclerotiorum from cultures grown on a liquid malt-yeast medium at 20°C.

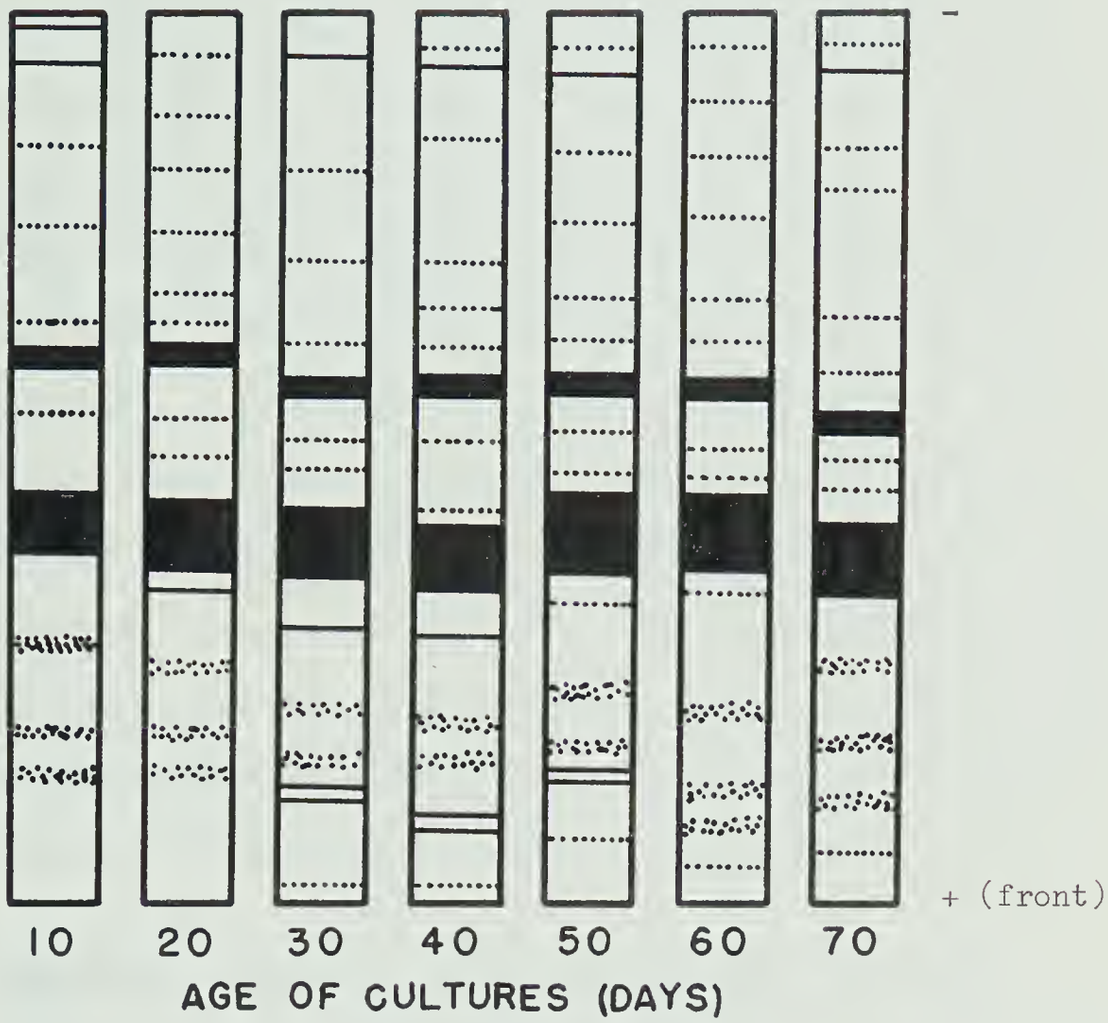


Fig. 15. Disc gel electrophoretic patterns of soluble proteins of sclerotia of S. sclerotiorum from cultures grown on a synthetic-agar medium at 15°C.

malt-yeast medium at 20°C. The three prominent bands present in the center of the gels from sclerotia of 10-, 20-, 30-, and 40-day old cultures were similar to those found for sclerotia cultured on the liquid malt-yeast medium. In sclerotia from 50-, 60-, and 70-day old cultures, only two of these prominent bands were present. There was a considerable difference in numbers and patterns of lightly stained bands for sclerotia cultured on the synthetic medium and the liquid malt-yeast medium. A noticeable difference was the presence of two lightly stained bands between the central prominent bands, whereas three bands were observed in sclerotia from cultures grown on the liquid malt-yeast medium. It appears that even after sclerotia reached their full size, changes occur within the sclerotia. These changes, although readily observed in sclerotia grown on the liquid malt-yeast medium, are not as evident for sclerotia cultured on the synthetic medium.

(b) Comparative studies of S. sclerotiorum and Sb_3 and Sb_4

The protein patterns observed in extracts from apothecia and sclerotia from which the apothecia were removed, for S. sclerotiorum, Sb_3, and Sb_4 are shown in Fig. 16. For comparision, the protein patterns of sclerotia prior to their being placed in water are also shown (procedure for producing apothecia--see page 5). The protein patterns of sclerotia prior to germination of isolates Sb_3 and Sb_4 were very similar, but the protein patterns differed considerably from that of S. sclerotiorum. However, in the sclerotia from which the apothecia were removed, the protein pattern for each isolate was considerably different from the others. In general, the numbers of bands in the sclerotia of S. sclerotiorum with the apothecia removed were slightly lower, but in Sb_3 and Sb_4 they were approximately half that present

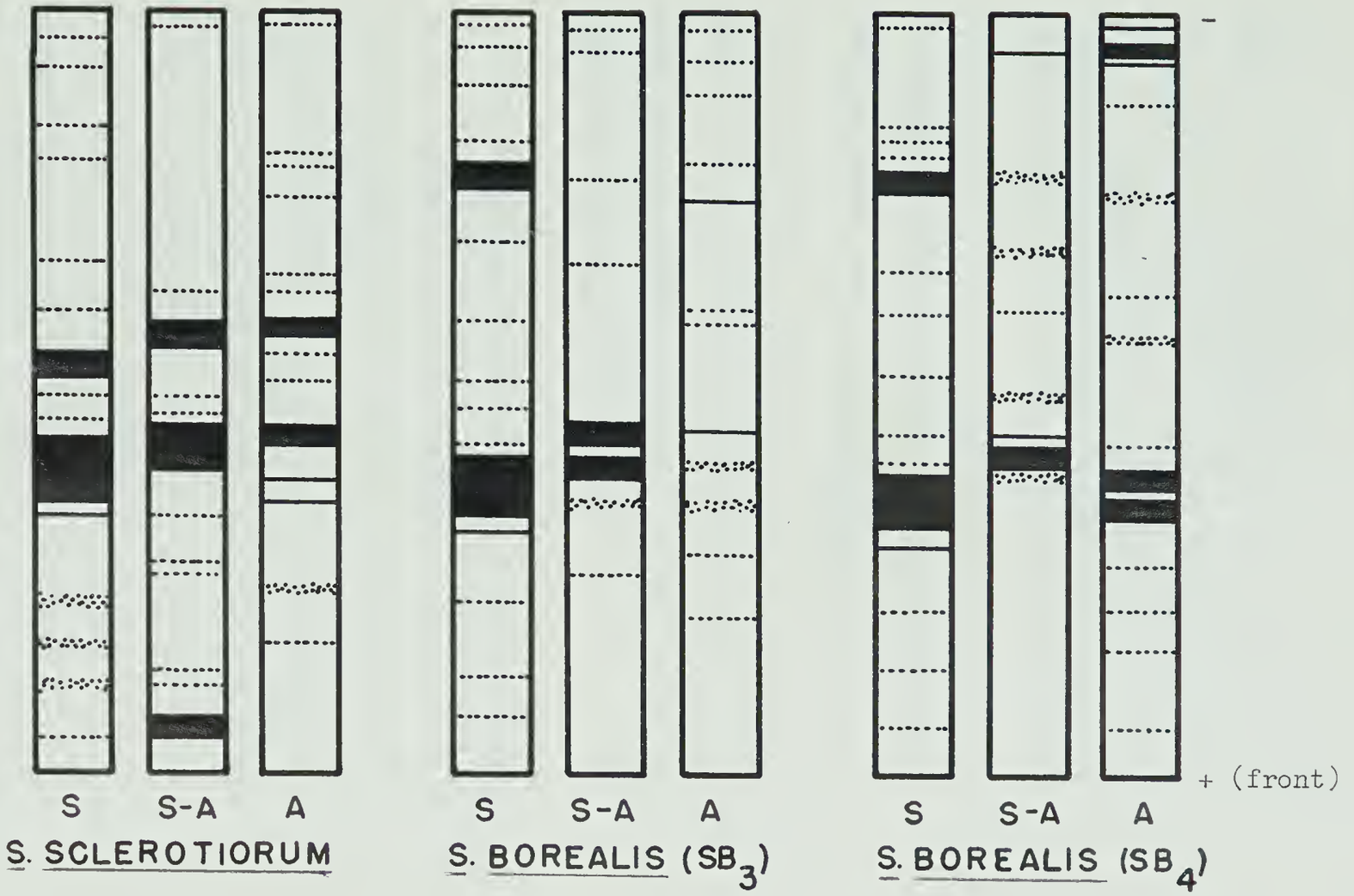


Fig. 16. Disc gel electrophoretic patterns of soluble proteins of mature ungerminated sclerotia (S), germinated sclerotia with the apothecia removed (S-A), and apothecia (A) of S. sclerotiorum and two isolates of S. borealis (Sb_3 and Sb_4).

in mature and ungerminated sclerotia.

Glynn and Reid (1969) considered that electrophoretic patterns can be used for taxonomic purposes if all cultural conditions were identical. In their electrophoretic studies of a low temperature basidiomycete, Sekhon and Colotelo (1969) found that different ages of cultures resulted in differences in protein patterns. Incubation temperature was also found to alter the protein pattern for this low temperature basidiomycete (Sekhon and Colotelo, unpublished data). In comparing protein patterns of S. sclerotiorum with Sb_3 and Sb_4 it should be noted that S. sclerotiorum was cultured on a different medium and at a different temperature than were the S. borealis isolates. However the Sb_3 and Sb_4 isolates were grown under identical conditions. Therefore, the differences observed can be considered to be due to differences between the isolates.

(c) Peroxidase and polyphenol oxidase isozymes of S. sclerotiorum

Patterns for peroxidase isozymes of extracts from sclerotia of S. sclerotiorum of various ages, produced on the synthetic medium at 15°C, germinated sclerotia with the apothecia removed, and apothecia, detected by benzidine-2HCl and pyrogallol reagents, are shown in Figs. 17 and 18 respectively. There was a differential stain reaction with the benzidine-2HCl and pyrogallol reagents. Peroxidase isozymes were detected in all gels stained with pyrogallol but none with benzidine-2HCl in sclerotia from 50-, 60-, and 70-day old cultures. In young sclerotia, the isozyme patterns were similar with both reagents except for sclerotia from 10-, and 20-day old cultures which had one additional band occurring in the upper third of the gels when benzidine-

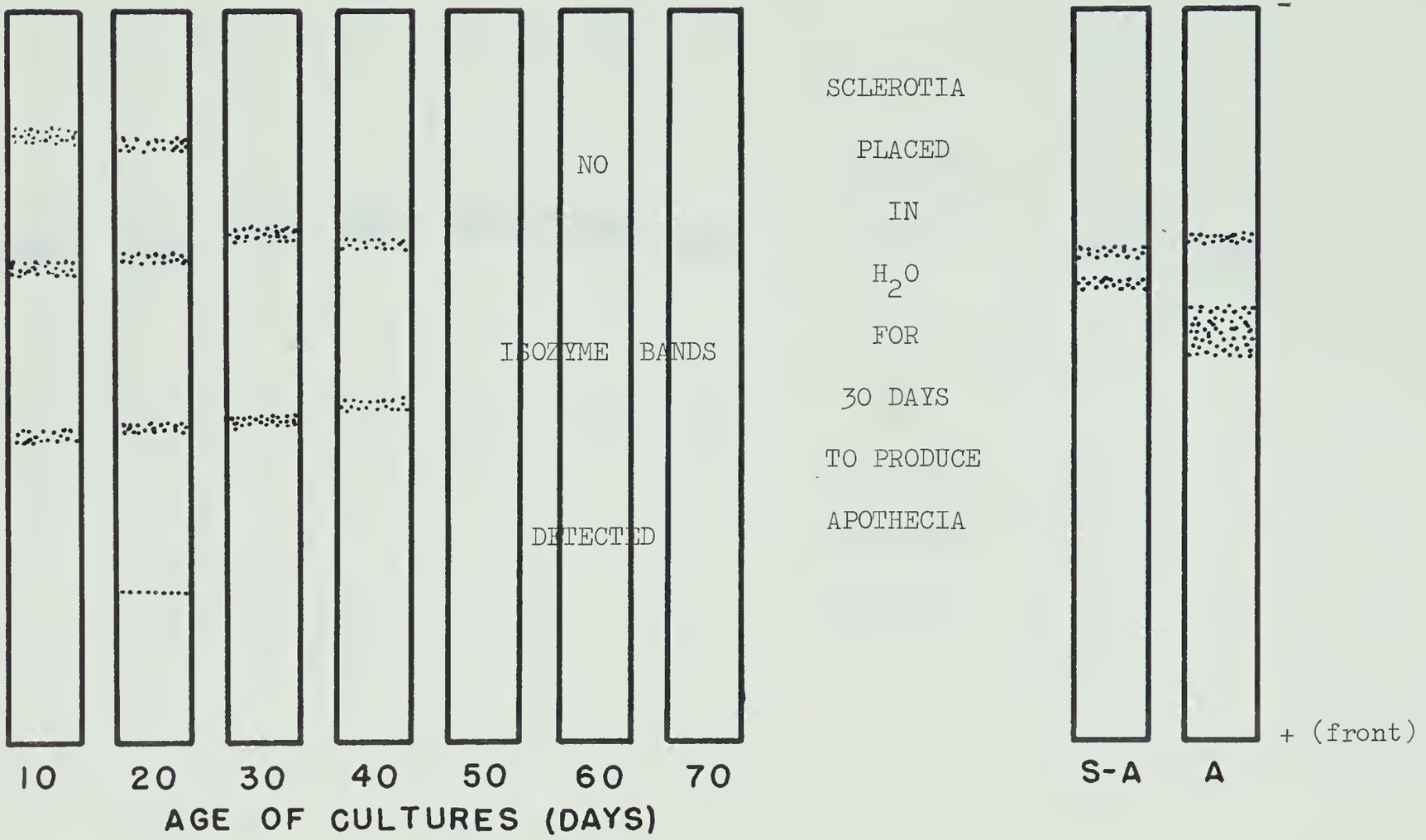


Fig. 17. Disc gel electrophoretic patterns of peroxidase isozymes of ungerminated sclerotia, germinated sclerotia with the apothecia removed (S-A), and apothecia (A) of S. sclerotiorum. Cultures were grown on a synthetic-agar medium at 15°C. Reagent for detecting peroxidase isozymes was benzidine-2HCl.

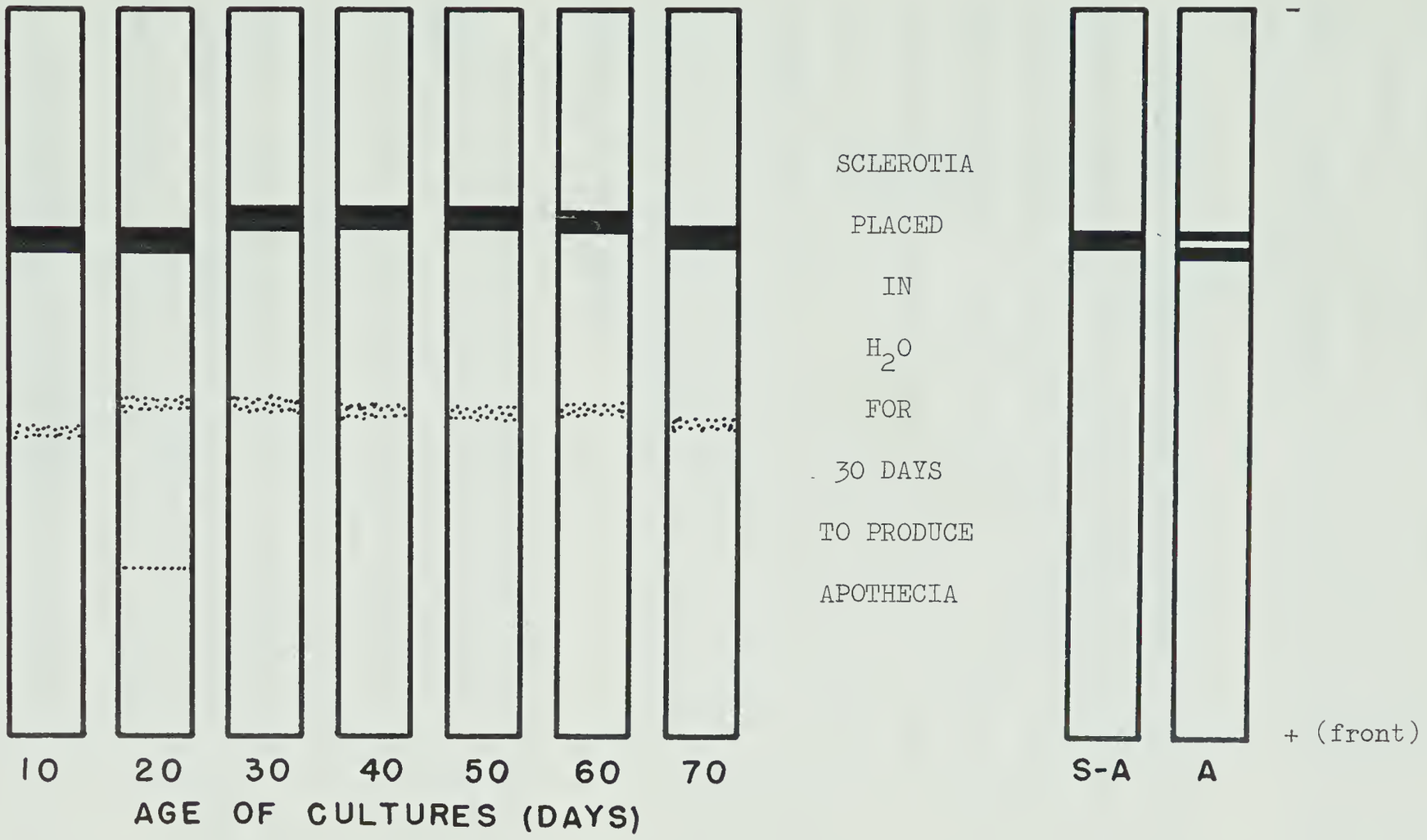


Fig. 18. Disc gel electrophoretic patterns of peroxidase isozymes of ungerminated sclerotia, germinated sclerotia with the apothecia removed (S-A), and apothecia (A) of S. sclerotiorum. Cultures were grown on a synthetic-agar medium at 15°C. Reagent for detecting peroxidase isozymes was pyrogallol.

2HCl reagent was used. Relative to age of culture, a very faint band was observed in the lower third of the gels stained with benzidine-2HCl and pyrogallol reagents for sclerotia harvested from 20-day old cultures.

Extracts of sclerotia with the apothecia removed have one band which was common to that found in younger and ungerminated sclerotia, and this band was detected with both reagents. However with benzidine-2HCl an additional band was observed. In apothecia there were two bands detected with benzidine-2HCl and one of which had a similar E_f value to that observed in ungerminated and germinated sclerotia, while the other band which was wide and diffuse did not correspond to bands in the other gels. With pyrogallol, two distinct bands were observed and the E_f values were similar to one another and to the heavy bands observed in other gels.

The differences in peroxidase isozymes of sclerotia of S. sclerotiorum stained with benzidine-2HCl and pyrogallol reagents may be due to the differential ability of these reagents to detect other oxidases. For example, pyrogallol not only detects peroxidase but it also detects catalase (Saunders et al. 1964). The benzidine-2HCl reagent is also sensitive to oxidation by other oxidizing agents (Saunders et al. 1964). Other differences may be due to substrate specificity.

The polyphenol oxidase isozymes in extracts of ungerminated sclerotia, sclerotia with the apothecia removed, and apothecia of S. sclerotiorum, are shown in Fig. 19. The greatest number of isozymes were detected in sclerotia from 20-day old cultures. Sclerotia of 10-, 20-, 30-, 40-, 50-, 60-, and 70-day old cultures had identical patterns

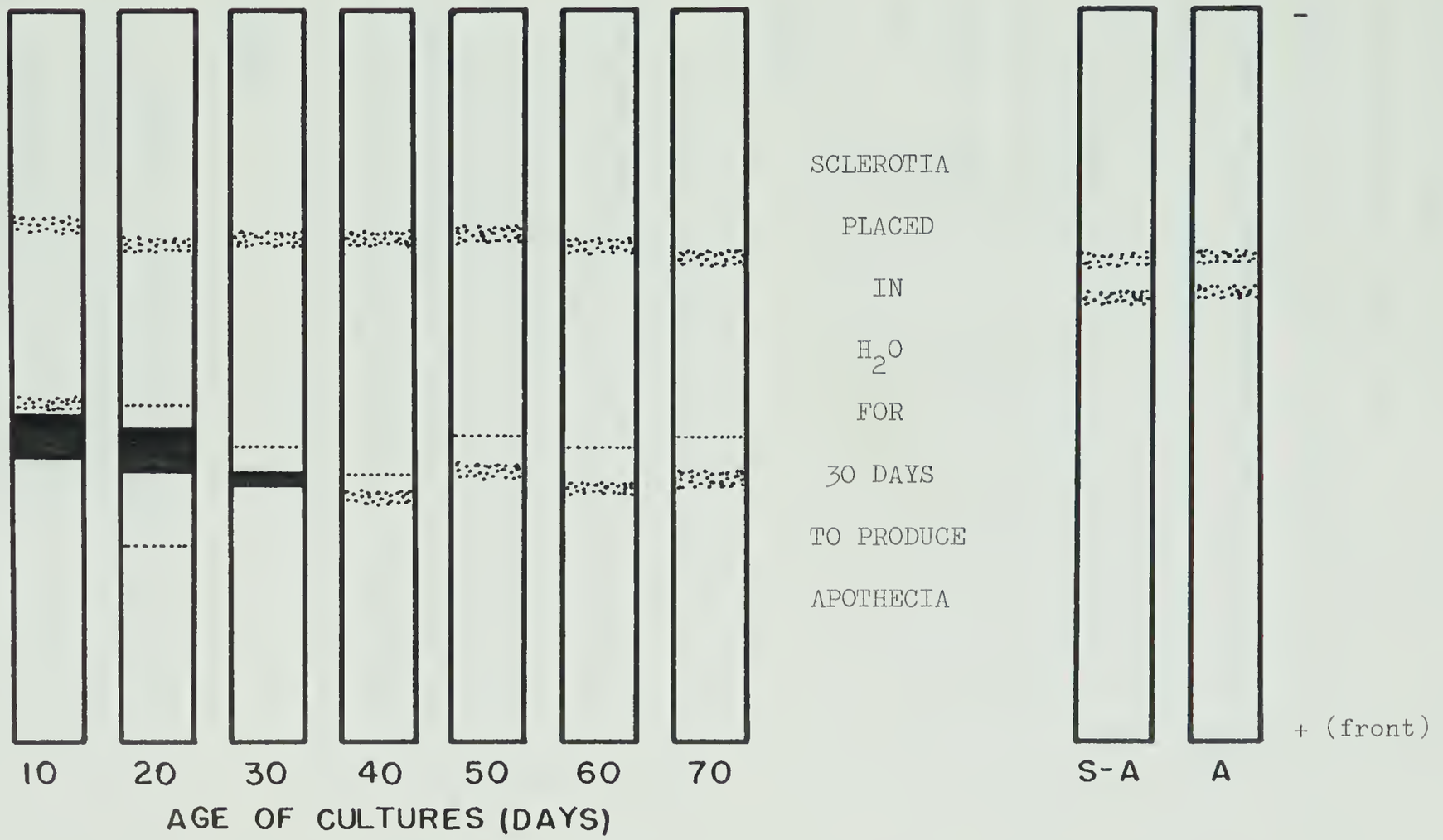


Fig. 19. Disc gel electrophoretic patterns of polyphenol oxidase isozymes of ungerminated sclerotia, germinated sclerotia with the apothecia removed (S-A), and apothecia (A) of S. sclerotiorum. Cultures were grown on a synthetic-agar medium at 15°C. Reagent for detecting polyphenol oxidase isozymes was l-dihydroxyphenyl-alanine (l-DOPA).

for three isozymes. However, in extracts of 20-day old cultures, there was an additional isozyme band. Activity of the third isozyme band of sclerotia from 10-, 20-, and 30-day old cultures resulted in l-DOPA being oxidized to a greater extent than by the other bands. Oxidation was also greater than that for similar isozyme bands in subsequent extracts.

Germinated sclerotia with the apothecia removed, and the apothecia had similar polyphenol oxidase isozyme patterns, however the patterns differed from those observed in ungerminated sclerotia. Isozyme bands detected in the lower half of the gels of ungerminated sclerotia were not observed in germinated sclerotia with the apothecia removed or apothecia.

A greater intensity of stain uptake by the polyphenol oxidase isozymes was observed in the younger sclerotia than in the older sclerotia even though the same amount of protein, as determined by the Lowry et al. (1951) method, was used for each gel. This greater activity of polyphenol oxidase in younger sclerotia could possibly be due to the involvement in pigment synthesis. Peroxidase may also be involved in pigment synthesis as was found by MacMillan and Brandt (1966) in Verticillium.

Makkonen and Pohjakallio (1960) found that the cortex tissue of sclerotia plays an important role in the resistance of sclerotia of S. sclerotiorum to parasitic fungi. Sclerotia, which developed new cortical tissues rapidly after being damaged, were more resistant to parasite attack than those that replaced the cortical tissue more slowly. Makkonen and Pohjakallio also noticed that the cut surface darkened rapidly after being cut. This pigmentation required adequate aer-

ation indicating that polyphenol oxidase was involved. When the original cortex of sclerotia was removed, added catalase was found to hasten the development of a new cortex.

From visual comparisons of the polyacrylamide gels, it appears that polyphenol oxidase activity is highest during the stages of sclerotial pigmentation. Peroxidase, which can act as an oxidizing enzyme, may be involved in pigmentation. The results for peroxidase, stained with benzidine-2HCl indicate their presence during growth and development of sclerotia, but no bands were present on the gels when the sclerotia were fully developed. However peroxidase isozymes were again detected in germinated sclerotia. Therefore, peroxidase may be involved in some biochemical aspects of growth and development of apothecia.

D. AMINO ACID ANALYSIS OF SCLEROTIA AND APOTHECIA

Qualitative results for free and bound amino acids of ungerminated sclerotia, germinated sclerotia with the apothecia removed, and apothecia are shown in Tables II and III respectively.

(a) Free amino acids

The free amino acids, alanine, arginine, glutamic acid, glycine, leucine and/or isoleucine, lysine, serine, threonine, and valine were present in extracts of ungerminated sclerotia at all stages of development, germinated sclerotia with the apothecia removed, and also apothecia. Asparagine and tryptophan were present in ungerminated sclerotia, germinated sclerotia with the apothecia removed, but not in the apothecia. Aspartic acid, present in sclerotia from 10-, 30-, and 60-day old cultures was not detected in sclerotia from 20-, 40-, 50-, and

	AGE OF CULTURES (DAYS)						
	10	20	30	40	50	60	70
alanine	D**	D	D	D	D	D	D
arginine	D	D	D	D	D	D	D
asparagine	D	D	D	D	D	D	D
aspartic acid	D	ND*	D	ND	ND	D	ND
DOPA	ND	D	D	D	D	D	D
glutamic acid	D	D	D	D	D	D	D
glutamine	D	D	D	D	D	D	D
glycine	D	D	D	D	D	D	D
leucine and/or isoleucine	D	D	D	D	D	D	D
lysine	ND	D	D	D	D	D	D
phenylalanine	D	ND	ND	ND	ND	D	D
serine	D	D	D	D	D	D	D
threonine	D	D	D	D	D	D	D
tryptophan	D	D	D	D	D	D	D
tyrosine	ND	ND	D	D	ND	ND	ND
valine	D	D	D	D	D	D	D

SCLEROTIA PLACED IN H_2O FOR 30 DAYS TO PRODUCE APOTHECIA

S-A	A
D	D
D	D
D	ND
ND	D
ND	ND
D	D
ND	D
D	D
D	D
ND	D
D	D
D	D
D	ND
ND	ND
D	D

*ND = not detected **D = detected

Table II. Free amino acids of ungerminated sclerotia cultured on a synthetic-agar medium at 15°C, germinated sclerotia with the apothecia removed (S-A), and apothecia (A).

70-day old cultures nor in germinated sclerotia with the apothecia removed, but was present in apothecia. Glutamine was present in ungerminated sclerotia at all stages of development and in apothecia, but was not detected in the germinated sclerotia with the apothecia removed. Phenylalanine was present in ungerminated sclerotia from 10-, 60-, and 70-day old cultures and in apothecia but was not detected for sclerotia from 20-, 30-, 40-, and 50-day old cultures nor in germinated sclerotia with the apothecia removed. Tyrosine was detected only for sclerotia from 30-, and 40-day old cultures and proline was not detected in the free amino acid extracts.

The free amino acids are considered to be a reservoir for synthesis of proteins and other metabolic processes (Holden 1962). Therefore changes in the free amino acid pool could be due to changes in proteins and/or enzymes. A change in free amino acids may be required before apothecia can be produced. In Sclerotinia trifoliorum, Sproston and Pease (1957) found that the use of thermoperiods during the growth of sclerotia subsequently increased apothecia production. During these thermoperiods, Obreiter and Sproston (1964) found that threonine content decreased noticeably. In sclerotia of S. sclerotiorum, the changes in amino acids may be due to a maturation process. During this maturation period certain amino acids accumulate, thus giving the sclerotia a reserve of amino acids required to form enzymes required during germination. The relation between free amino acids and maturation could be resolved by a quantitative analysis of free amino acids of sclerotia during development, maturation, and germination to form apothecia.

(b) Bound amino acids

The bound amino acids, alanine, arginine, aspartic acid, glutamic acid, glycine, leucine and/or isoleucine, phenylalanine, proline, serine, threonine, and valine were found in all stages of ungerminated sclerotia, germinated sclerotia with the apothecia removed, and apothecia. Lysine was not detected in ungerminated sclerotia of 20-, 40-, and 50-day old cultures but was found in the other stages of ungerminated sclerotia as well as in sclerotia with the apothecia removed, and apothecia. Except in sclerotia from 10-day old cultures and apothecia, tyrosine was found in all stages of development of ungerminated sclerotia and germinated sclerotia with the apothecia removed. Fewer qualitative changes were observed in the bound amino acids than in the free amino acids.

The data for bound amino acids does not indicate significant qualitative differences except for the absence of tyrosine in apothecia. As with free amino acids, more significance to data would be obtained by quantitative analyses of bound amino acids of sclerotia during development, maturation, and germination to form apothecia.

E. FACTORS AFFECTING APOTHECIA PRODUCTION

Results for the effects of the liquid malt-yeast and the synthetic medium on apothecia production are presented on page 25. The effects of plant extracts, glucose concentration, 1,3-dichloropropene, and light and darkness on apothecia production are presented in this section.

	AGE OF CULTURES (DAYS)						
	10	20	30	40	50	60	70
alanine	D**	D	D	D	D	D	D
arginine	D	D	D	D	D	D	D
aspartic acid	D	D	D	D	D	D	D
glutamic acid	D	D	D	D	D	D	D
glycine	D	D	D	D	D	D	D
leucine and/or isoleucine	D	D	D	D	D	D	D
lysine	D	ND*	D	ND	ND	D	D
phenylalanine	D	D	D	D	D	D	D
proline	D	D	D	D	D	D	D
serine	D	D	D	D	D	D	D
threonine	D	D	D	D	D	D	D
tyrosine	ND	D	D	D	D	D	D
valine	D	D	D	D	D	D	D

*ND = not detected **D = detected

SCLEROTIA PLACED IN H_2O FOR 30 DAYS TO PRODUCE APOTHECIA

S-A	A
D	D
D	D
D	D
D	D
D	D
D	D
D	D
D	D
D	D
D	D
D	D
D	ND
D	D

Table III. Bound amino acids of ungerminated sclerotia cultured on a synthetic-agar medium at 15°C, germinated sclerotia with the apothecia removed (S-A), and apothecia (A).

(a) Plant extracts

The numbers of apothecia, produced from sclerotia grown on the synthetic medium and germinated in plant extracts, were similar to the numbers produced in controls (control--sclerotia germinated in H_2O) (Table IV). However, when sclerotia were produced on the synthetic medium supplemented with the plant extracts, except in the case of the sunflower extract, and allowed to germinate in H_2O, the numbers of apothecia were almost nil (Table V). Using extracts of the aboveground parts and roots of barley, and roots of rape, the sclerotia did not germinate to produce stipes. When extracts on the aboveground parts of rape were used sclerotia produced several (5) apothecia. The numbers of apothecia produced when extracts of the aboveground parts of sunflower were used were as great as observed for controls.

Many years ago, it was found that sclerotia which had formed on sunflower, hollyhock, and pumpkin formed apothecia, but sclerotia from squash did not (Young 1934, 1936). In support of Young's studies, Purdy (1956) found that sclerotia produced in cultures grown on potato-dextrose agar did not form very many apothecia, whereas those sclerotia from cultures grown on autoclaved celery produced many more apothecia. In the results of the study, it was found that the type of medium during the growth of sclerotia (Table V) was more important than materials in which the sclerotia were placed for germination to form apothecia (Table IV).

(b) Glucose concentration

The effects of glucose concentration on production of apothecia are shown in Table VI. Apothecia were not produced from scle-

CONCENTRATION OF PLANT EXTRACTS (PERCENT)	NUMBER OF APOTHECIA PRODUCED PER 12 SCLEROTIA
CONTROL (H_2O)	15
MATURE BARLEY (ABOVEGROUND PARTS)	
1.5	14
0.75	14
0.15	10
0.015	11
MATURE BARLEY (ROOTS)	
1.5	17
0.75	12
0.15	19
0.015	13
MATURE RAPE (ABOVEGROUND PARTS)	
1.5	11
0.75	12
0.15	20
0.015	19
MATURE RAPE (ROOTS)	
1.5	17
0.75	13
0.15	16
0.015	25
IMMATURE SUNFLOWER (ABOVEGROUND PARTS)	
1.5	16
0.75	17
0.15	19
0.015	20

Table IV. Production of apothecia from sclerotia of S. sclerotiorum placed in H_2O (control) and different concentrations of plant extracts. Sclerotia taken from 70-day old cultures were grown on a synthetic-agar medium at 15°C.

rotia grown on the malt-yeast-agar medium supplemented with 1.0% and 2.0% glucose. However, 25 apothecia per 20 sclerotia were produced from sclerotia grown on the malt-yeast-agar medium with 3.0% glucose. This indicated that C/N ratios of nutrients during the growth of the sclerotia are important for subsequent apothecia production. Although the C/N ratio has been found to be important for sclerotia production (Turian 1966) this aspect has not been dealt with previously for apothecia production.

Factors, such as plant extracts and glucose, change the C/N ratio of the growth medium. Mature barley plants, as used in these experiments, are low in nitrogen and would increase the C/N ratio, and thus could inhibit subsequent apothecia production. Sunflower (immature) probably did not alter the C/N ratio and therefore has little effect on apothecia production. The possibility exists that those plant residues which inhibit apothecia production contain a natural inhibitor or in the case of sunflower a stimulant which could increase apothecia formation.

(c) 1,3-dichloropropene

The number of apothecia produced from sclerotia, treated with 0.1% 1,3-dichloropropene prior to germination in H_2O is shown in Table VII. The number of apothecia produced from sclerotia treated with 1,3-dichloropropene was almost four times that of controls. These results confirm the observations made by Paptyka and Mai (1958) who found that sclerotia in soils treated with 1,3-dichloropropene produced more apothecia than sclerotia in untreated soils. The mechanism or mode of action of 1,3-dichloropropene on the metabolism of sclerotia is unknown.

PLANT EXTRACTS	NUMBER OF APOTHECIA PRODUCED PER 12 SCLEROTIA
CONTROL (SYNTHETIC MEDIUM)	22
MATURE BARLEY (ABOVEGROUND PARTS)	0
MATURE BARLEY (ROOTS)	0
MATURE RAPE (ABOVEGROUND PARTS)	5
MATURE RAPE (ROOTS)	0
IMMATURE SUNFLOWER (ABOVEGROUND PARTS)	23

Table V. Production of apothecia from sclerotia of S. sclerotiorum from 70-day old cultures grown on a synthetic-agar medium (control) and a synthetic-agar medium supplemented with plant extracts (0.375%) and then germinated in H_2O. Cultures were grown at 15°C.

GLUCOSE (%) ADDED TO MALT-YEAST-AGAR MEDIUM	NUMBER OF APOTHECIA PRODUCED PER 20 SCLEROTIA
1.0	0
2.0	0
3.0	25
0 (CONTROL)	0

Table VI. Production of apothecia from sclerotia of S. sclerotiorum grown on a malt-yeast-agar medium supplemented with different concentrations of glucose at 15°C. Control--malt-yeast-agar medium without glucose.

TREATMENT	NUMBER OF APOTHECIA PRODUCED PER 50 SCLEROTIA
1,3-DICHLOROPROPENE	11
H_2O (CONTROL)	3

Table VII. Apothecia production of S. sclerotiorum at 15°C from mature sclerotia treated with 1,3-dichloropropene for 3 days prior to germination in H_2O.

(d) Effects of light and dark

In the controls in the dark, long stipes were formed (Fig. 20a), but in the light normally expanded apothecia were produced (Fig. 20b). These results are in agreement with Henderson (1962) and Bedi (1962).

-Chemical treatments. The chemical treatments which included H_2O_2, peroxidase, H_2O_2 plus peroxidase, and IAA resulted in stipe formation from sclerotia germinated in the dark (Fig. 21 a-d) but in the light normal apothecia were produced. The addition of H_2O_2, peroxidase, and polyphenol oxidase respectively, to cultures kept in the dark, resulted in stipes similar to those obtained for dark controls. The addition of H_2O_2 plus peroxidase, to cultures kept in the dark, resulted in branching of apothecial stipes (Fig. 21c). With IAA the stipes were much shorter than controls (Fig. 21d) and slightly branched although in Fig. 21d this branching is not evident. The addition of catalase to cultures kept in dark and light resulted only in mycelial growth.

The branching of apothecial stipes in the dark when H_2O_2 plus peroxidase was added to the cultures is an interesting phenomenon. A similar branching effect was observed in decapitated stipes of S. sclerotiorum by Henderson (1962). She found that unbranched stipes were normally formed, and she considered that this type of growth was regulated by an auxin synthesized in the apex of the stipe. When the apex was removed, little or no auxin would be produced and branching of the stipe resulted. According to Henderson, "It is doubtful, therefore, if the phenomenon can be likened to that of the apical dominance which occurs in higher plants." Cartile (1965) suggests that the auxin

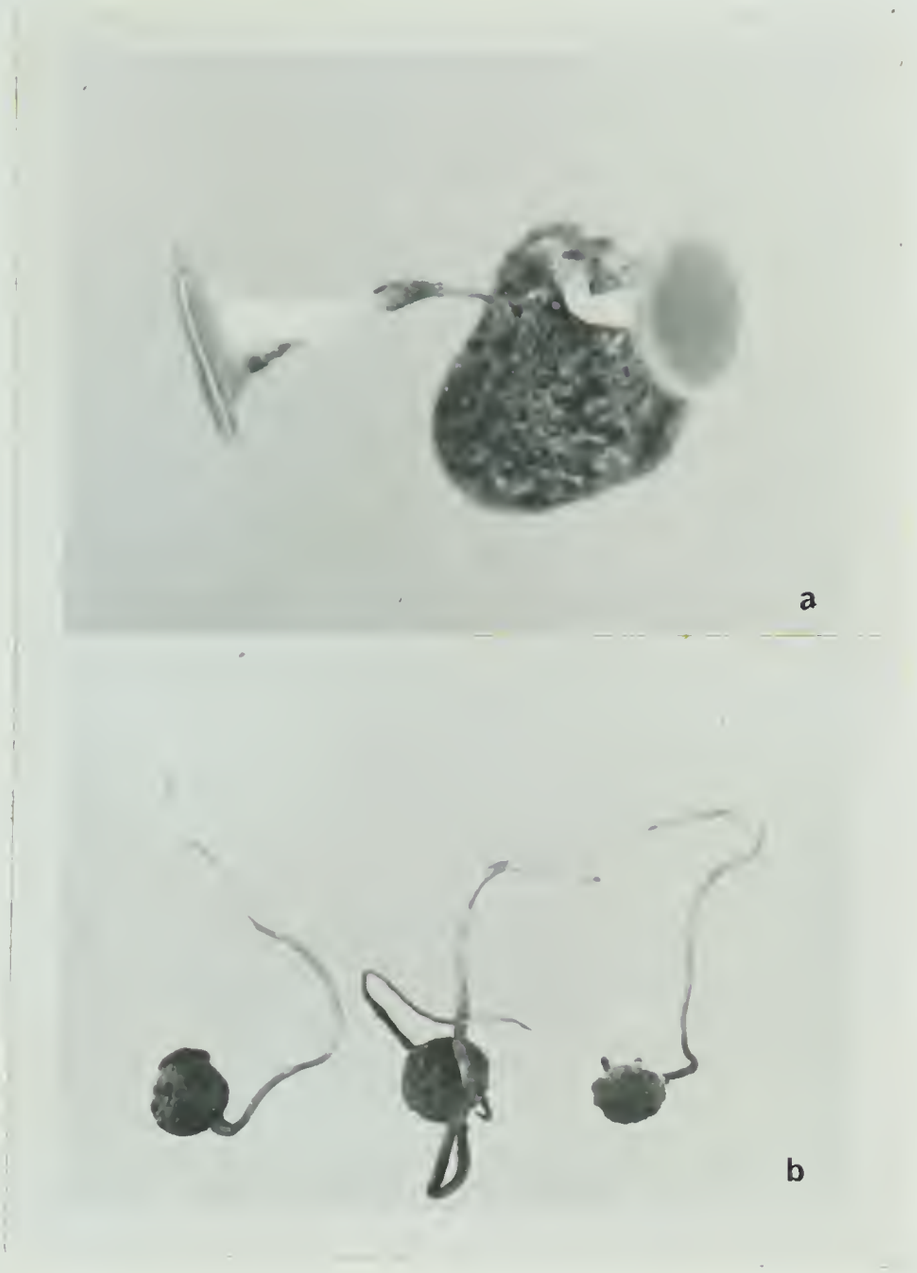


Fig. 20. Apothecia (a) produced by mature sclerotia of S. sclerotiorum germinated in water in the light and apothecial stipes (b) produced in the dark. Incubation temperature was 15°C. Magnification (a) 5X, and (b) 1.5X.

in fungi is IAA and that it undergoes photoreduction in the light. If the auxin in apothecial stipes is IAA, then it can be oxidized to an inactive form as is suggested by Galson et al. (1953) for IAA in peas. He suggests that the mechanism of IAA oxidation is as follows: light activates a flavoprotein which produced H_2O_2. Peroxidase plus H_2O_2 oxidize IAA to an inactive form. In the apothecial stipes of S. sclerotiorum the inactivation of IAA by peroxidase plus H_2O_2 would account for the branching which was observed. Therefore, the elimination of IAA by this method would give the same results as the decapitation experiments by Henderson.

The use of various chemical treatments in the dark were not able to substitute for light in the production of apothecia of S. sclerotiorum. Of interest is the induction of asexual sporulation of Sclerotinia fructogena by IAA in the dark (Khan 1966). When IAA was added to cultures in the dark sporulation was similar to that observed in the light.

F. PIGMENTATION OF SCLEROTIA

Of the compounds tested, sodium diethyldithiocarbamate (dieca) was the only one which inhibited pigmentation and decreased dry weight of sclerotia and increased yield of mycelium (Fig. 22). At 10^{-3} M concentration, pigmentation of the sclerotia was delayed until the cultures were 15 days old and at 5×10^{-3} M, pigmentation was delayed until the cultures were 20 days old. Also, the sclerotia in 2-day old cultures were only light grey in color and did not turn black as the cultures aged. Normally pigmentation of sclerotia begins in the 6-day old cultures and sclerotia are black by the eighth day. The inhibition of growth of sclerotia was proportional to the concentration of dieca.

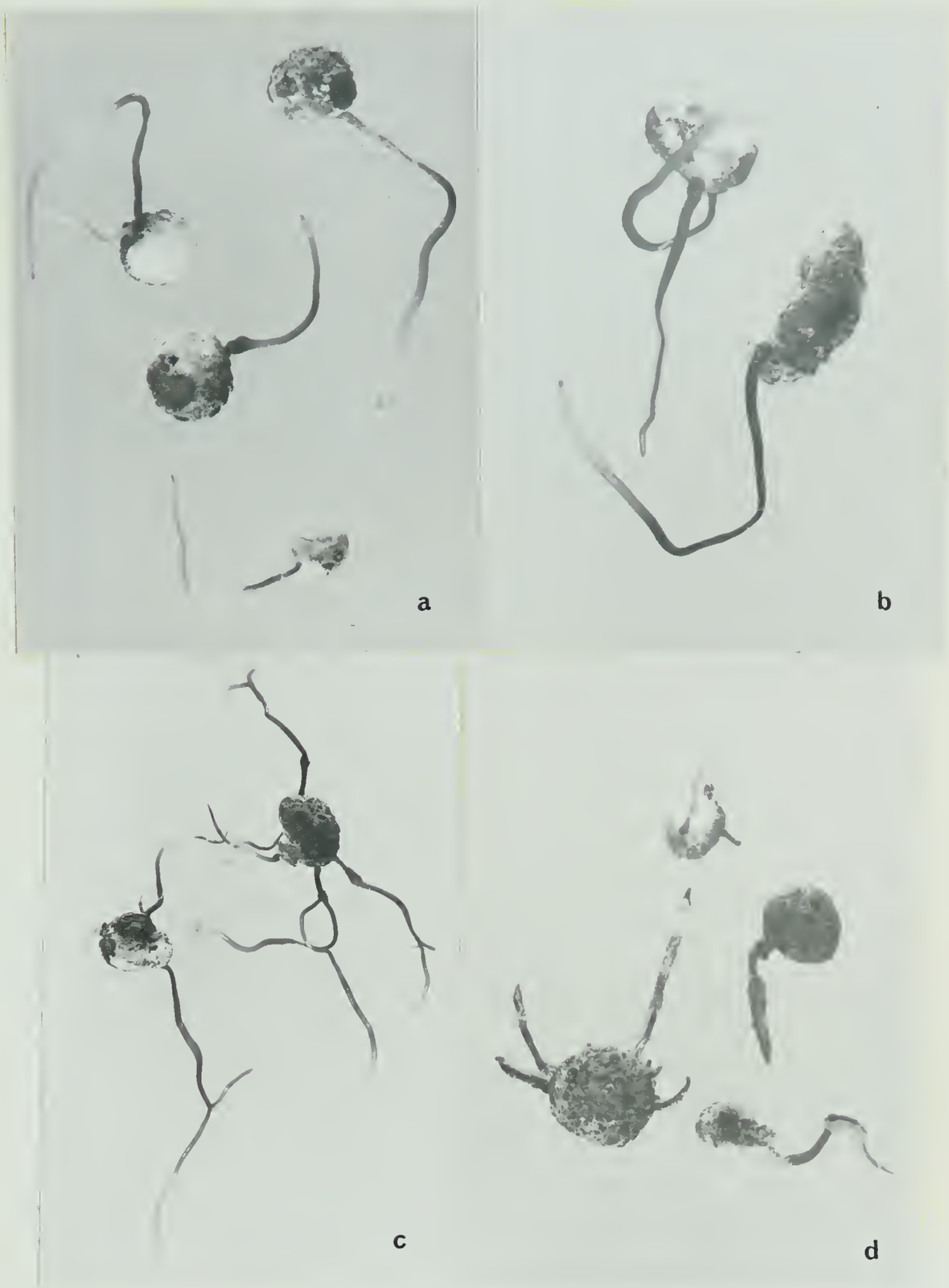


Fig. 21. Mature sclerotia of S. sclerotiorum germinated in solutions containing H_2O_2 (a), peroxidase (b), H_2O_2 plus peroxidase (c), and IAA (d). Incubation temperature was 15°C. Magnification (a) 2X, (b) 2.5X, (c) 1.5X, and (d) 2X.

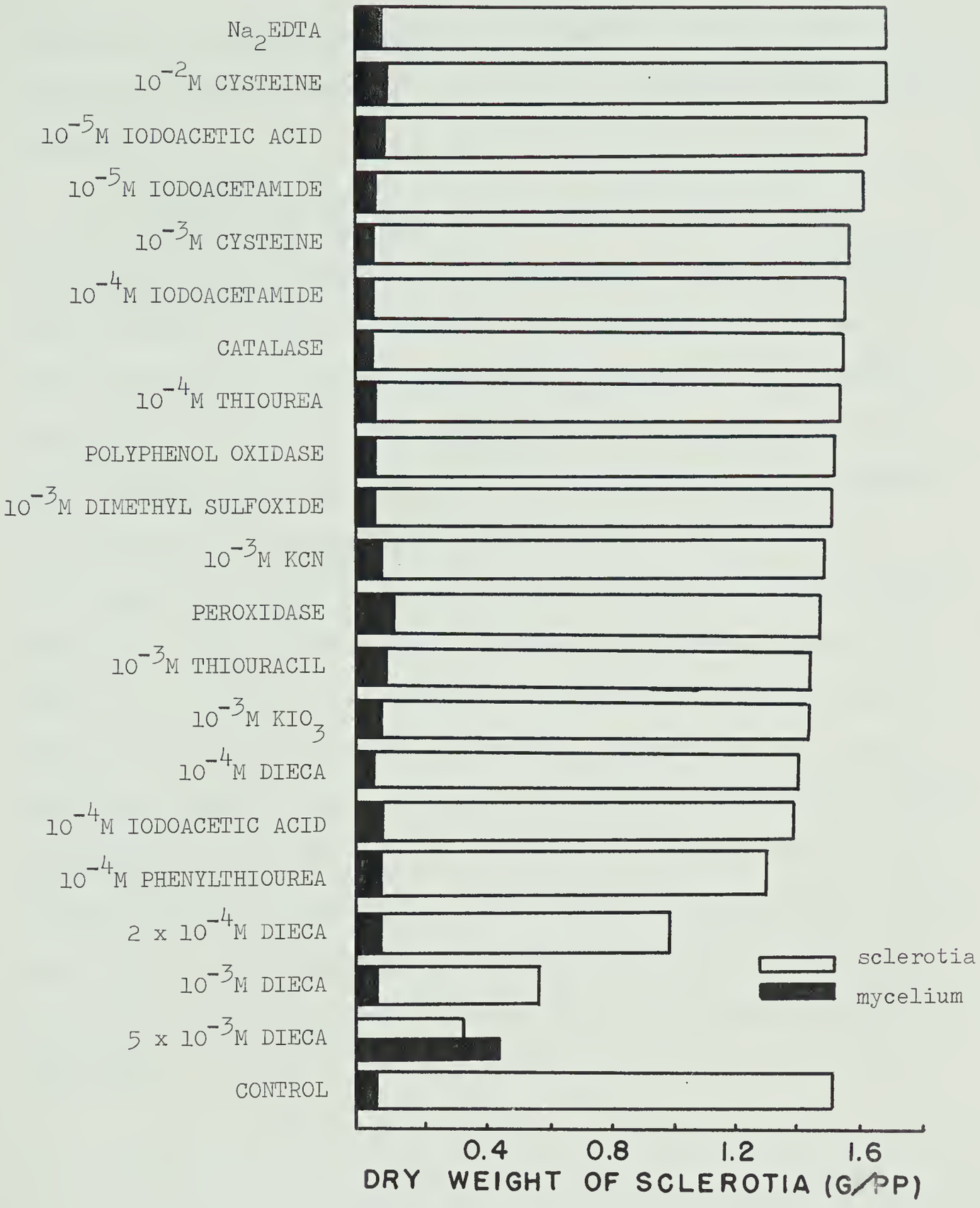


Fig. 22. Dry weights of mycelium and sclerotia from 20-day old cultures of S. sclerotiorum grown on a liquid malt-yeast medium supplemented with various compounds used to inhibit sclerotial pigmentation. Cultures were incubated at 20°C.

Although not recorded numerically, the numbers of sclerotia were less and smaller than the controls. At 5×10^{-3} M, greater mycelial growth was observed.

The pigments extracted from several fungi by Bloomfield and Alexander (1967) were found to be melanin-like. They suggested that the resistance of fungal structures (e.g. sclerotia) was correlated with the presence of melanin or melanin-like materials. The pigment extracted from sclerotia of S. rolfsii was found to have characteristics of melanin (Chet et al. 1967). The indications are that pigment of sclerotia of S. sclerotiorum is melanin-like. The synthesis of this pigment was inhibited by dieca which is known to inhibit melanin synthesis. Dieca chelates copper and some other metals (James 1953). This is in contrast to the findings of Bonner (1957) who found that dieca can be used to differentiate copper proteins from other metal-containing proteins. Since growth of sclerotia was inhibited and mycelial growth was stimulated by this compound, indications are that other enzymes involved in induction and development of sclerotia are partially or completely inhibited and if these same enzymes are also involved in pigmentation, then pigmentation may also be partially or completely inhibited.

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